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Abstract

DNA obtained from cells deposited by handling an item is referred to as 'touch' DNA. Touch DNA can play an important role in forensic science since it is an extremely common and challenging form of DNA evidence. Frequently, touch DNA samples are low templates and often are mixtures, making them typically one of the most difficult types of evidence to interpret and distinguish contributors, leading to a decrease in the success rate of casework. Short tandem repeat (STR) analysis is a standard method to estimate the number of contributors and DNA yield for each contributor; however, there are some limitations. Applying a non-destructive pre-DNA analysis technique that provides information about the touch DNA may improve the casework process. Antibody probes to cholesterol-based hormones such as testosterone and dihydrotestosterone (DHT), and proteins including cytokeratins (CK) inside epidermal cells, have been demonstrated to produce a personalized signature for human touch cell populations. Testosterone and DHT are feasible markers for touch samples since the epidermis is a target tissue for the hormones. CK is a suitable target since cytokeratins are a primary component of this terminal cell type. Both hormone and cytoskeletal targets are stable for long periods, making them logical candidates to act as markers for the differentiation of cell populations. In this study, testosterone and DHT and CK binding efficacy among humans was evaluated using swabs of epithelial cells collected directly from each volunteer's hands. Fluorescently-tagged antibodies were bound to targets in the cells and analyzed with flow cytometry. The results demonstrated that testosterone and DHT-specific antibodies were able to distinguish between single source touch samples or mixtures comprised of more than one person. And CK staining results demonstrated that anti-CK can differentiate cell populations between two individuals. The order of touching an item was predicted by antibody staining intensity as well as a correlation between fluorescent signal intensity and DNA content. The quantitation data showed that the DNA amount correlated with the shift in fluorescence in histograms, indicating that this technique could be used to predict DNA yield before DNA extraction and quantitation. We concluded that antibody hybridization targeting the testosterone/DHT and CK targets has the potential to serve as a non-destructive technique prior to STR analysis to enhance the probative value of biological evidence.

Key words:

Forensic Science, Touch DNA, DNA Mixtures, Testosterone, Dihydrotestosterone, Cytokeratins, Antibody Hybridization, Flow Cytometry

Contents

Abbreviations

DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
STR	Short Tandem Repeat
CE	Capillary Electrophoresis
LT-DNA	Low Template DNA
CODIS	Combined DNA Index System
PG	Probabilistic Genotyping
T	Testosterone
DHT	Dihydrotestosterone
CK	Cytokeratins
DFS	Virginia Department of Forensic Science
PBS	Phosphate-Buffered Saline
RCF	Relative Centrifugal Force

Chapter 1. Introduction

1.1 Touch/Trace DNA

Locard's Exchange Principle states that “Every contact leaves a trace”. When there is contact between two items, there will be an exchange of microscopic material[1]. DNA from skin cells including nucleated epithelial cells, shed corneocytes, and cell-free DNA transferred by handling from a person to an object, is frequently referred to as touch DNA[2,3]. Items that are associated with a crime might have sufficient DNA which can be analyzed and linked to a person who left DNA on the object. Touch DNA is one of the most common forms of evidence that is requested for DNA analysis[4], which plays an important role in forensic science[5,6].

Touch DNA from skin cells is primarily from keratinocytes (stratum corneum layer of the epidermis), nucleated epithelial cells, or residual cell fragments **(Appendix Figure 1)**[5]. Epithelial cells from the stratum corneum layer are primarily keratinized and have lost their nuclei. DNA has been detected in keratinocytes[7]. However, studies have demonstrated that there is no correlation between the number of keratinocytes and the amount of DNA[8]. Nucleated epithelial cells and residual cell fragments have also been reported to be sources of touch DNA[5]. Most touch DNA obtained from objects is low copy, and the quality and quantity of DNA recovered are affected by the donor's shedder status, sex, age, activities, and environment[9,10]. Low template DNA can result in allele/full locus drop-out and frequently leads to obtaining unusable DNA profiles, which is an issue in forensic cases[11].

1.2 Forensic DNA Mixtures

A DNA mixture refers to evidence that consists of DNA from two or more individuals. Evidence that is touched by multiple individuals or DNA/epithelial cells co-transferred from another donor may contribute to touch DNA mixture profiles[5]. DNA mixtures are common and also the most challenging types of evidence for DNA crime laboratories[12]. Due to the different proportions of DNA in a mixture profile, the profile of the minor contributor who has lower DNA content may exhibit allele or full locus drop-out, leading to the development of an incomplete DNA profile. Complex mixtures contain DNA from three or more contributors, resulting in an increased frequency of allele drop-out and shared alleles, making them difficult to interpret data, which decreases the value of the evidence and the success rate to solve the case[13,14].

1.3 Methods of DNA Analysis in Forensic Science

Short tandem repeats (STR) profiling is a technique that utilizes fluorescent STR primers and PCR to make millions of copies of targeted short repeated sequences, which vary in number from person to person. Due to these repeats varying in numbers from person to person at specific loci in DNA, these sequences can be separated by product size with capillary electrophoresis and finally, to utilize specialized genotyping software in order to obtain a forensic DNA profile. Because of variation in the number of repeats among humans at each locus and the utilization of the 20 Combined DNA Index System (CODIS) core loci as a requirement for the generation of a DNA profile, STR profiling can have a high power of discrimination when comparing DNA samples between two or more[15].

Currently, STR analysis is the most common method to estimate the number of contributors and predict the amount of DNA for each contributor in touch DNA mixture samples. While total DNA within a sample can be estimated using DNA quantitation technologies, the current quantitation systems are hard to distinguish the number of contributors and their respective contributions to the sample[13]. There are numerous commercial STR typing kits, including PowerPlex® Fusion (Promega) and Globalfiler® (Applied Biosystems); however, most of them have limitations for typing mixture samples, with many profiles resulting in complicated interpretation[16,17]. For example, it can be hard to distinguish minor contributors' true alleles from artifacts such as stutter[14]. Moreover, even though peak height imbalance at heterozygous loci may distinguish major/minor contributors in the mixture STR profile, allele/locus drop-in/out frequently occurs when the minor's DNA contribution is below 5%[14]. STR analysis is not suitable for severely degraded DNA samples such as when DNA fragment length is below 300bp and lacks template DNA for amplification[18]. Even probabilistic genotyping software systems that can facilitate mixture analysis to derive the most probable genetic profiles of the contributors to a mixture, may still provide false negative/positive result with very challenging samples [19-21]. These obstacles can make a definitive interpretation difficult[22].

1.4 Testosterone and Dihydrotestosterone

Cholesterol-based androgen hormones such as testosterone (T) and dihydrotestosterone (DHT) are involved in the development of male reproductive tissues and promote secondary sexual characteristics[23]. Testosterone is mainly secreted by the testes, and DHT is a product of testosterone which is synthesized by 5α-reductase[24]. Approximately 10% of testosterone is converted to DHT in target tissues of testosterone[25]. Hormones are mainly known to be

metabolized in the liver by binding with the androgen receptor. Testosterone and DHT are also able to be secreted and synthesized in the skin in order to form the epidermal barrier and sebum [26]. Because testosterone is found in both men and women and skin is a target tissue, testosterone and DHT are reasonable targets for studying epidermal cell touch samples. Moreover, cholesterol-based hormones have been demonstrated to be stable up to 40 years when stored at -20°C[27,28]. Another research also displayed that testosterone can stay stable without degradation for a long period. It remains 90% of concentration when stored in a fridge for 5 months[27]. Studies have also demonstrated that due to sex, age, activity, and environmental factors, the amount of hormone inside epidermal cells can differ between individuals. For example, study showed that testosterone in males is 10 times higher than in females[29]. Resulting in personalized, if not unique, hormone binding signatures[30,31]. There are currently no markers available for presumptive or confirmatory identification of epidermal skin cells, unlike seminal fluid, blood, and saliva, which are all also common types of forensic evidence[32]. These factors make testosterone and DHT strong candidates to act as markers to distinguish individuals in epithelial cells from touch evidence in the forensic field.

1.5 Cytokeratins

Cytokeratins (CK) are cytoskeleton proteins that are specific to epithelial cells[33]. CK plays an important role in forming the intracellular network of intermediate filaments, and are organized into 20 families (CK-1 to CK-20) according to their molecular weights[34]. The abundance of each cytokeratin class within a cell is determined by the cell's differentiation status, and by factors such as types of epithelium and age[35]. For example, CK-1,2,6,10 and 16 are highly expressed in keratinocytes, while CK-4 and CK-13 are expressed in nucleated epithelial cells[36]. Additionally,

research has demonstrated that the AE1 binding site antibody detects CK-10, CK-14 to 16, CK-19, while the AE3 binding site antibody targets CK-1 to 8[37]. CK have been proposed to be a personalized signature marker in sloughed epidermal cells[35,38,39]. However, there is little research using CK to differentiate epithelial cell populations. CK are highly expressed in epithelial cells, and are abundant in the stratum corneum layer of epidermal cells that are a significant component of touch samples, thus, they have the potential to be used as a marker in a wide range of forensic applications including touch evidence[33,33].

1.6 Antibody Hybridization and Flow Cytometry

Antibody hybridization is a method used to measure the abundance of specific proteins in biological samples by utilizing probes that are specific to the target antigen/protein[40]. The attachment of fluorescent dyes onto antibodies is a common strategy used to easily detect the amount and location of target molecules in biological samples, including cells[41]. For example, fluorescein (FITC), is a commonly used fluorescent tag. The excitation maximum of FITC is at 488 nm wavelength and emits at a wavelength of 519 nm, producing a green color where the fluorescent tag antibodies bind. With different excitation maximum and emission wavelengths, antibodies tagged with different fluorescent dye colors of fluorescent can be applied to do the multiplex testing[42].

Flow cytometry is a technique that provides quantifiable data from a sample by detecting the physical and chemical characteristics of cells that flow one cell at a time past the detector[43]. It is widely used in biology research and clinical science[44]. During the process, cells are suspended in a buffer or liquid and injected into the flow cytometer instrument. Cells are passed one cell at a

time through a laser beam. Forward Scatter light (FSC) is used to measure the size of the cell, while Side Scatter light (SSC) is for analyzing the internal complexity of the cell. Moreover, fluorescently conjugated antibodies in the cells may emit fluorescence and these signals are then collected by a multicolor-detector and then analyzed **(Appendix Figure 2)**[45]. With the ability to collect thousands of events per second and equipped with separate fluorescence channels to analyze multi-targets[44,46], flow cytometry has versatility, sensitivity, and the quantitative capabilities to measure cells by scattered lights rapidly. Additionally, it analyzes target proteins/hormones in cells by fluorescent emissions effectively in a non-destructive manner.

1.7 Benefits of Analyzing Touch DNA with a Non-Destructive Method

Touch DNA is a challenge in forensic DNA analysis due to the predominance of mixtures and low levels of DNA[11]. Low template DNA leads to allele and locus drop-out, producing less informative STR profiles[11]. Probabilistic genotyping could facilitate mixture analysis; however, the system is lacking validation[19]. STR analysis still is the cornerstone of forensic DNA analysis, including estimating the number of contributors and generating profiles for human identification[15]. This process starts with DNA extraction, which is a destructive and irreversible process[47]. Therefore, having a non-destructive technique that provides as much information about the sample as possible before STR profiling may inform interpretation in an unbiased manner. This pre-DNA information, particularly about a cell type (epidermal skin cells) that has no presumptive or confirmatory tests available, may provide a more informed approach to downstream DNA analysis.

1.8 Research Goals

Previous findings have demonstrated that antibody probes, such as HLA-antigen in blood samples, can be used to differentiate cell populations in mixture samples in forensic applications, however, there are no published reports on distinguishing epidermal cell populations from touch cell mixtures[48,49]. Recently, our lab has found that testosterone and DHT, as well as CK (AE1/AE3 binding site) antibodies may be able to differentiate cell populations in touch samples from each sole source contributor due to different binding efficacy of antibodies in different individuals[30,50]. Investigating the binding efficacy of antibodies for each contributor and determining whether there is a correlation between estimated and measured DNA yield may increase the resolution in mixture samples.

According to previous studies[30,50] and the results reported here, analysis of touch samples with fluorescent-tagged target-specific antibodies using flow cytometry may allow for the prediction of the number of contributors to a mixed sample. DNA yield in touch mixture samples may be predicted by antibody binding efficacy in a non-destructive manner.

1.9 Impact

Our project investigates a new pre-DNA-profiling method that can predict touch DNA in epithelial cell mixtures rapidly and in a non-destructive manner. This new approach uses fluorescent-tagged antibodies that recognize intra-cellular molecules in order to estimate contributor number and DNA content while preserving the DNA from touch evidence before cell destruction and STR analysis. We developed a new technique has the potential to increase the

information available for mixture touch evidence prior to DNA profiling, which may assist with sample triaging as well as data analysis and interpretation.

1.10 Hypothesis and Specific aims

Hypothesis:

Fluorescent-tagged testosterone- and dihydrotestosterone- specific antibodies and CK-specific antibody can serve as a pre-DNA profiling technique in touch DNA evidence.

Aim 1:

To estimate the number of contributors in touch DNA mixture samples by testosterone and dihydrotestosterone specific antibody hybridization.

Aim 2:

To compare the result between antibody hybridization and DNA yield to determine whether a correlation exists.

Aim 3:

To study cytokeratins binding efficacy in touch DNA samples and for this, I used cytokeratins specific antibody hybridization.

Chapter 2. Materials and Methods

2.1 Sample Collection

All samples were collected under Institutional Review Board (IRB) review and approval (VCU-IRB approved protocol# HM20000454_CR6).

2.1.1 Creating Single Source Samples

Each cotton swab was moistened in 200 µL of sterile diH_2O. Epithelial cells were collected from donors by swabbing both their palms directly. Swabs were allowed to air dry at room temperature, and epithelial cells were eluted by soaking swabs in 1.5 mL PBS (Quality Biology, 119-069-131) for 10 minutes, and vortexed for 30 seconds. Swabs were squeezed with a tweezer and were removed prior to centrifuging at 14,000 RCF for 10 minutes. The supernatant was aspirated and 700 µL of 5% Triton X-100 (Thermo Scientific, 85111) was added directly to the pellet to resuspend the cells. After incubation at room temperature for 15 minutes, epithelial cells were washed with 700 µL of PBS twice and centrifuged at 14,000 RCF for 10 minutes for each wash. Finally, the supernatant was aspirated and cells were eluted in 500 µL of PBS.

Samples were then divided half; 250 µL for antibody hybridization, and 250 µL for DNA analysis **(Figure 1, panel A)**.

2.1.2 Creating 1:1 2-Person Mixtures

Epithelial cells were collected from two donors separately using the collection and elution protocol for single source procedure. Cell counts were measured by flow cytometry, and a 1:1

mixture sample with 5,000 cells for each donor was created in a new 1.5 mL tube. The mixture sample and remaining single source samples were hybridized to antibodies as described and followed by analysis using flow cytometry.

2.1.3 Creating Mixtures for Predicting Touch Sequence

Two 50 mL falcon conical centrifuge tubes (Genesee Scientific, 28-108) were each held by one person for 5 minutes, and then switched to be held by the other person following the same procedure. Item #1 was touched by person 2, then person 1 with their left hands, and item #2 was touched by person 1, then person 2 with another hand. Swabs were moistened in 200 μL of diH_2O, and epithelial cells on the tubes were collected by swabbing the tubes directly. Swabs were then processed as described for the single source procedure **(Figure 1, panel B)**.

2.2 Non-Destructive Methods

2.2.1 Antibody Hybridization

All eluted epithelial cells were centrifuged at 14,000 RCF for 10 minutes, and all the supernatant were removed. Added 700 μL of blocking buffer (2% bovine serum albumin (GeminiBio, 700-100P) in PBS) at room temperature for 1 hour. Each sample was divided equally into 3 tubes (unstained, isotype stained, antibody stained). The isotype group of epithelial cells was probed with 1.9 μg IgG1 isotype control antibody (Novus Biologicals, NBP1-96849) to serve as a negative control; the antibody stained group epithelial cells was probed with 1.9 μg anti-testosterone (Novus Biologicals, NB 100-62943F) and 4.1 μg anti-DHT (MyBioSource,

MBS2042441), 2.225 µg AE1 CK (Novus Biologicals, NBP2-62221F), or 2.225 µg AE1/AE3 CK (Novus Biologicals, NBP2-33200F) in 1 mL fresh blocking buffer at 4℃ overnight. Antibody-stained epithelial cells were centrifuged at 14,000 RCF for 10 minutes to remove the supernatant, and washed twice with PBS. Cell pellets were re-suspended in 350 uL of cell staining buffer (BioLegend, 420201), and the presence of the antibody targets was detected by flow cytometry (Luminex, Guava® easyCyte™).

2.2.2 Flow Cytometry

Epithelial cells were passed through a 100-um filter (Fisher Scientific, NC1432771) before injection into the flow cytometer for detection. After cleaning capillaries with the cleaning solution (Luminex, 4200-0140) in the cleaning cycle and completing the easyCheck protocol (Luminex, 4500-0025) for the Guava® easyCyte™ (Luminex), samples were analyzed by the Guava® easyCyte™ using a 488 nm Laser (FITC channel) at a high speed and low photomultiplier 1.0 voltage for 3 minutes. Data were analyzed and histograms were generated using the FlowJO® software v10. Fluorescence intensity (GRN-B-Log mean) was expressed as relative fluorescence units (RFU).

Previously, our lab used a high photomultiplier voltage set at level 4.0 in flow cytometry to analyze the fluorescent signal of fluorescent-tagged antibodies. However, the high voltage resulted in some fluorescent signals falling outside of the linear range of detection and reduced accuracy[51]. One sample's fluorescent signal was out of range and generated unusable values **(Supplementary Figure 1, panel A).** We found that a low photomultiplier voltage setting at level

1.0 could collect both low and high fluorescent signals from the anti-testosterone and DHT antibodies (**Supplementary Figure 1, panels A, B, D, and E**).

2.2.3 Cell Staining

Antibody-stained cells were stained with DAPI (Invitrogen, R37606), a DNA intercalating agent, in order to observe DNA location. DNA and testosterone/DHT/CK locations were observed under Confocal Microscope (Nikon C2 Plus two-laser; at 405 nm and 488nm). Cells and images were analyzed using the NIS-Elements Viewer 5.21 software.

2.3 DNA Analysis

2.3.1 DNA Extraction (Organic Extraction)

Epithelial cells: Eluted epithelial cells were centrifuged at 56,000 RCF for 10 minutes at room temperature. The supernatant was removed and the pellet was retained. Lysis buffer (proteinase K enzyme (20 mg/mL) and stain extraction buffer in a 1:40 ratio) was added into the tube. Tubes were vortexed for 15 seconds, followed by incubation at 56℃ for 2 hours in the dry bath incubator.

Buccal cells: Whole swabs were soaked in lysis buffer at 56℃ for 2 hours. Swabs were placed into Spin-EASE baskets inserted into the 1.5 mL tube, followed by centrifuging at 56,000 RCF for 5 minutes. Swabs and the Spin-EASE basket were then discarded.

Eluted DNAs in lysis buffer were mixed with phenol-chloroform-isoamyl alcohol (25:24:1) in a 1:1 ratio. After vortexing the tubes for 15 seconds and centrifugation at 56,000 RCF for 3

minutes, supernatants were transferred to Microcon® 100 concentrators (Millipore MRCF0R100). And followed by centrifugation at 56,000 RCF for 10 minutes. The flow-throughs were discarded and 200 µL of nuclease-free water was added to Microcon® 100 concentrators followed by centrifugation at 14,000 RCF for 15 minutes. The 1.5 mL tubes were discarded and 30 µL of TRIS-EDTA (TE^{-4}) were added to the Microcon® 100 concentrator filters. Microcon® 100 concentrators were inverted into a new 1.5 mL tube and centrifuged at 14,000 RCF for 5 minutes to elute DNA. The eluted DNA was stored at -20℃ until use.

2.3.2 Real-time quantitative PCR (RT-qPCR)

DNA was quantitated by the PowerQuant® System (PQ5002; Promega Corp., Madison, WI). DNA quantitation standards were created in a 25-fold serial dilution series for three concentrations starting with undiluted (50 ng/µL). 2 µL DNA standard series or DNA samples were added to an optical 96-well plate (VWR, 82050-698), and 18 µL of DNA quantitation master mix that included 10 µL master mix, 7 µL nuclease-free water, and 1 µL primer for each sample. The plate was sealed with clear film (VWR, 82050-994) and reactions were performed using QuantStudio™ 5 Real-Time PCR systems (Thermofisher Scientific, Waltham, MA) as described (ref PQ technical manual)[52]. Data were analyzed with PowerQuant™ Analysis Tool as described (PQ tech manual)[53].

2.3.3 STR Amplification

A target of 0.5 ng DNA was amplified using PowerPlex® Fusion (PowerPlex®, DC2402; Promega). Amplified reactions consisted of 7.5 µL of PCR mixed buffer and 5 µL of 0.5 ng DNA

or 2800M positive control DNA (PowerPlex®, DD710A) added into 0.1 amplification tubes. If DNA yield did not meet 0.5 ng/ µL, 5 µL of extracted DNA was amplified. Tubes were placed in a thermal cycler (Applied Biosystems 9700; Thermofisher) for 1 minute at 96℃, followed by 28 cycles of 10 seconds at 94℃, 1 minute at 59 ℃, and 30 seconds at 72 ℃. Reactions were incubated for 10 minutes at 60℃ and at 4 ℃ overnight. Amplified DNA was stored at -20℃.

2.3.4 Capillary Electrophoresis

Zero point five µL of WEN ILS 500 (Promega, DG5001), 9.5 µL of Hi-Di formamide (Applied Biosystems, 4311230), and 1 µL of amplified-DNA or allelic ladder (PowerPlex® Fusion, DG381B) were added to individual wells in optical 96-well plates (Applied Biosystems, N801-0560) and wells were covered with a plate septum (Applied Biosystems, 4315933). After briefly centrifuging, plates were heated at 95℃ for 3 minutes, and immediately transferred to a -20℃ freezer block for 3 minutes. Plates were inserted into a plate retainer and placed into a 24-capillary sequencing instrument (Applied Biosystems, 3500xl Genetic Analyzer). Both 12 second and 24 second injection data were collected as described in the Virginia Dept. of Forensic Science procedure manual[54].

2.3.5 STR DNA Profiling

CE results were analyzed with GeneMapper® ID-X software, v1.4. ILS size standard peaks, PowerPlex® Fusion allelic ladders, reagent blanks, negative controls, and positive controls were analyzed. An analytical threshold of 75 RFU was utilized and samples were reviewed and identified for artifacts, including pull-up, incomplete A nucleotide addition shoulder peaks, raised

baseline, spikes, and stutter. Stutter peaks were removed from the DNA profile when it was below the expected stutter percentages[54]. Observed alleles from each locus were recorded and a DNA profile was obtained for determining single source, mixture, or reference samples.

All DNA analysis methods were performed under the Virginia Department of Forensic Science's protocol[54].

2.4 Correlation Analysis

DNA yield and the average fluorescent intensity were normalized to cell counts data from FlowJo® software. The correlation coefficient between normalized fluorescent intensity and DNA yield was calculated with Excel (Microsoft Corp., Seattle, WA). Scatterplots were analyzed and drawn with Excel (Microsoft Corp., Seattle, WA).

Chapter 3. Results

3.1 Testosterone and DHT antibodies show specificity

To ensure that selected antibodies showed high-affinity binding specificity, epithelial cells were collected from the same donor, and separated into 3 groups; unstained, isotype negative control, and testosterone antibody. There was no significant difference between unstained and isotype control. Though auto-fluorescence was observed in unstained and isotype control groups, fluorescence intensity was significantly higher in the testosterone antibody stained group compared with unstained and isotype control groups, showing specific antibody binding **(Figure 2A)**. DNA staining showed that DNA was clumped in the unstained and antibody stained groups. The isotype group shows a classic epithelial cell that has lost the nucleus. Though a diffusion stained was observed, DNA still exists in the cells **(Figure 2A)**. The expression of FITC was significantly lower in unstained and isotype control stained epithelial cells compared to the testosterone and DHT antibody stained cells (FITC fluorescence intensity = 34.6 RFU (unstained) versus 41.8 RFU (isotype) versus 296 RFU (testosterone and DHT antibody stained)) **(Figure 2B)**. These results suggest that DNA existed in many epithelial skin cells. Additionally, testosterone and DHT antibodies showed a higher binding efficiency compared to isotype controls and can be easily resolved from auto-fluorescence signals in unstained cell populations.

3.2 Testosterone and DHT antibodies in a 1:1 2-person mixture

After confirming that the testosterone and DHT antibodies were specific for their targets, to test whether testosterone and DHT antibodies could distinguish individuals in mixture samples, a 1:1 2-person mixture was created. The peak shift for the testosterone and DHT antibodies staining group was significantly larger than the shift of the unstained and isotype control groups, indicating there was little non-specific binding in this combined-antibodies (FITC fluorescence intensity = 1646 RFU (unstained) versus 2140 RFU (isotype) versus 48876 RFU (testosterone and DHT antibodies stained)) **(Figure 3, panel A)**. Two separated peaks in the fluorescent histogram were observed **(Figure 3, panel A)**. The single source fluorescent histogram data showed that each peak of each single source sample was aligned with the peak position in the mixture profile **(Figure 3, panels B and 3C)**. These results show that due to the difference in expression of testosterone and DHT in skin cells among individuals (or some other specific target recognized by the antibodies), there is a potential to distinguish individuals in skin cells mixture profiles as well as provide single source confirmation.

3.3 Testosterone and DHT antibody in single source samples

To further study the abundance of target molecules in cell populations, six contributor cell populations were collected and analyzed. Six fluorescence intensity values were obtained **(Figure 4, panels A-F)**. Also, 6 different distributions in the merged-fluorescent histogram were observed **(Figure 4, panel G)**, though patterns showed overlapping distributions (**Supplementary Figure 2, panel A**), there was a difference in mean fluorescence (FITC fluorescence intensity = 605 RFU (A24) versus 439 RFU (B16) versus 805 RFU (D67) versus 846 RFU (H71) versus.1584 RFU (I66) versus 296 RFU (N27)). This indicates that testosterone and DHT antibodies may distinguish

individuals and the resulting fluorescence histogram distribution could be a proxy for the number of contributors in the epidermal skin cell mixture.

3.4 Testosterone and DHT antibodies in a 2-person touch mixture sample

To investigate whether testosterone and DHT antibodies can be used in mixture samples, 2-person touch mixture samples were created. Data demonstrated that 2 clear peaks were observed in the mixture fluorescent histogram **(Figure 5A)**. However, there were no obvious differences between single source or 2-person mixture profiles if the number of contributors was unknown in all 4 mixture fluorescent histograms **(Figure 5A and 5B)**. When the cell count is low, the shoulder peaks in the single source cell population histogram may cause ambiguity in estimating the number of contributors (a shoulder peak was observed in single source when the cell count is 1910 versus a clear single peak in the single source when the cell count is 2963) **(Figure 4, panels F and E)**. Furthermore, the cell count of each peak in the histogram was affected by the order of touching the item. The last- person to handle the substrate had a higher cell count, and the former-touched person has a lower cell count for each item **(Figure 5, panel A, Items #1 and #2; Figure 5, panel B Item #1 and #2)**. The merged fluorescent histograms showed that for each person, the highest peak position (highest cell count) in the staining patterns was the same **(Figure 5A Merge and 5B Merge)**. Taken together, these results indicate that the distribution of fluorescence across the cell population following antibody binding could change with the varying numbers of contributors, and the peak heights could change by the order of contributors contacting the item. This technique may be able to predict the sequence of contributors contacting the item in a mixture sample prior to DNA analysis.

3.5 Correlation between DNA content and fluorescent intensity after testosterone and DHT antibody staining

Tests were performed to determine whether the binding efficiency of fluorescently-tagged antibody probes could be used to predict DNA yield prior to conventional DNA extraction and amplification techniques. Correlation tests between fluorescent intensity and DNA yield were performed. Once again it confirmed that testosterone fluorescently tagged antibodies could differentiate individuals **(Figure 6, panel A)**. Combining testosterone antibody with DHT antibody increased the separation fluorescent shift, and increased the resolution of fluorescent peak signal **(Figure 6, panels B and C)**. Each donor's DNA concentration was quantified (**Figure 6, panels D-F)**. Furthermore, the correlation between normalized testosterone fluorescent intensity and normalized DNA yield showed over a 95% correlation coefficient between fluorescent intensity and DNA yield (n = 5) **(Figure 6, panel G)**. With the combination of testosterone and DHT antibodies, the correlation coefficient increased to 98% (n = 4) and 99% (n = 6) **(Figure 6, panels H and I)**. However, there is always variance between individuals. After removing an outlier sample, the correlation between two variables increased. The correlation coefficient rose from 43% to 95% (Figure 6, panel G), and from 93 % to 99% (Figure 6, panel I). These results suggest that fluorescently-tagged testosterone antibody could be used to predict DNA yield in touch DNA evidence before DNA extraction, and adding DHT antibody to the anti-testosterone antibody could increase the effect and accuracy.

3.6 CK antibody in single source samples

To investigate the abundance of CK targets in touch epidermal cells, 4 donors' cell populations were collected and incubated with no antibody, isotype negative control, or CK antibody. However, there were no significant differences between unstained cells, isotype negative control stained cells, or CK antibody stained cells in the fluorescent histogram **(Figure 7, panels A, B, D, and E)**. Though four fluorescence intensity values were obtained and different peak shifts in the overlaid-fluorescent histogram were observed **(Figure 7, panels C and F)**, the unstained, isotype negative control histograms showed that the separation occurred without antibody staining, indicating the differences were not a consequence of CK antibody targeting. The resolution between the unstained group and CK antibody staining group increased with an increase of CK antibody added into touch epithelial cells (FITC fluorescence intensity = 36.7 RFU (unstained) versus 146 RFU (low concentration anti-CK) versus 267 RFU (mid concentration anti-CK) versus 628 RFU (high concentration anti-CK)) **(Figure 8, panels A-C)**. However, the expression of FITC in the isotype negative control group also increased with increasing amounts of the isotype antibody (FITC fluorescence intensity = 36.7 RFU (unstained) versus 65 RFU (low concentration anti-isotype) versus 91 RFU (mid concentration anti-isotype) versus 209 RFU (high concentration anti-isotype)) **(Figure 8, panels A, B, and D)**. To increase specific binding and decrease non-specific binding in CK antibody staining, the blocking buffer was switched from the commercial buffer (Thermo Fisher Scientific, #37515) to the 2% BSA blocking buffer. The data showed a significant decrease in the isotype negative control group (FITC fluorescence intensity = 71.8 RFU (commercial blocking buffer) versus 33.7 RFU (BSA blocking buffer)) **(Figure 9)**.

The data demonstrate that AE1/AE3 CK antibody binding produced a larger shift and better resolution between individuals (**Figure 10, panels D-F**) when compared to AE1 CK antibody

binding alone (**Figure 10, panels A-C)**. This result also showed the potential for CK to provide a personalized signature (FITC fluorescence intensity = 251 RFU (N27) versus 136 RFU (Z34)) **(Figure 10, panel F)**.

Chapter 4. Discussion

4.1 The role of testosterone and DHT antibodies in touch mixture samples

Estimating the number of contributors is important in epidermal cell mixture samples. STR analysis is the most common method, but due to shared alleles and frequently dropped alleles, an accurate number is not always possible[55]. Probabilistic genotyping software is a novel method to derive each unknown individual's genetic profile from mixture profiles, still, it is not fully mature[19]. The system is lacking consistency; different software packages can lead to obtaining different results from the same sample[20]. Anti-testosterone and DHT signals have been demonstrated to differ in epithelial cells among the human population[30,31]. Different fluorescence intensity values and two clear peaks in a 2-person mixture profile were observed in some mixtures **(Figure 4, panels A -F and Figure 5)**, but there were overlaps between individuals. Though adding anti-DHT along with anti-testosterone could increase the resolution **(Figure 6, panels A-B)**, overlapping patterns still exist, which could make a 6-person mixture profile looks like a 2-person profile **(Figure 4, panel G and Supplementary Figure 2, panel A)**. Estimating the number of contributors from this data alone is not obvious. To increase accuracy in estimating the number of contributors in mixture samples, add-effect mathematical modeling might be used to achieve this goal. We proposed that by using the add-effect mathematical modeling method, six peaks in the fluorescent histogram might be observed. However, additional analyses and experiments need to be performed to prove the concept (**Supplementary Figure 2, panels B and C**). Studies have demonstrated that testosterone and DHT are approximately 20 times greater in males than in females[56] and a higher fluorescent intensity signal in males was expected, but those differences

were measured for serum levels and differences in epidermal levels have not been reported[27]. Previously, our lab has demonstrated that sex alone does not predict the staining intensity of the anti-testosterone and DHT fluorescent antibodies[30,50] **(Figure 4, panels B-C)**. Taken together, anti-testosterone and DHT antibodies can be used to differentiate single source from mixture epidermal cell samples. To accurately estimate numbers and the sex of contributors in a sample, additional studies including adding more biomarkers are essential.

4.2 Testosterone and DHT antibodies may predict touch sequence and DNA yield before STR analysis

It can be important to build the timeline of the crime scene. There are many reports that the major contributor to a DNA profile from a touched item often is the last handler of the item[57,58]. We have demonstrated that the last user of the item displayed a higher fluorescent signal (greater shift to the right in the histogram) **(Figure 5)**, which aligns with the DNA analysis studies.

Low template DNA samples frequently result in allele or locus drop-in/out, sometimes resulting in obtaining an unusable DNA profile, which can be an issue for DNA analysis[22,47]. Here, we observed a high correlation between DNA content and fluorescent intensity **(Figure 6)**. However, we did not observe a strong correlation between DNA content and fluorescent intensity for the first experiment performed and proposed that this may be due to cell-free DNA (**Supplementary Figure 3**). Testosterone and DHT are expected to exist in the skin cells[26], therefore, extra-cellular DNA should be removed since while it adds to the total DNA content, the DNA is not correlated to the intra-cellular signal obtained by antibody binding. After removal of cell-free DNA, strong correlations between testosterone and DHT fluorescent intensity and DNA

yield were observed **(Figure 6, panels G-I)**. Our new findings suggest that testosterone plus DHT antibodies can predict the order of contributors touching the item as well as cellular DNA yield before STR analysis. Importantly, this fluorescent screening method is working in a non-destructive manner. By having the information of DNA quantity and quality prior to DNA analysis, low template DNA samples could be triaged in such a way as to enhance the likelihood of successful DNA profiling. This could impact the success rate for touch DNA evidence in the DNA forensic science field.

4.3 The role of CK antibody in epithelial cells

CK staining has been demonstrated to show a personalized signature for epithelial skin cells[35,38,39]. CKs have been classified into 20 families, and the AE1 CK antibody detects CK-10, CK-14 to 16, and CK-19; while the AE3 CK antibody targets CK-1 to 8[37]. Our results showed that the AE1 binding site for the CK antibody has a low binding specificity to epithelial cells (**Figure 10, panels A-C**). On the other hand, the AE1/AE3 binding site CK antibody can distinguish two individuals (**Figure 10, panels D-F**). In order to further study the role of CK antibody in epidermal cells, additional samples need to be collected to measure the variance between individuals.

4.4 Impacts of our findings in the forensic science field

STR analysis has been widely used in DNA forensic science[16,17]; yet, it is hard to distinguish contributors in mixture samples and low template DNA causes smaller peaks (lower RFU values) in the minor contributors which can lead to incomplete profiles. Probabilistic genotyping is being utilized in many laboratories, but the technology has some limitations in the

ability to resolve contributors to complex mixtures, particularly with contributor numbers greater than four [19-21]. Whole-genome sequencing (WGS) / next-generation sequencing (NGS) and single-nucleotide polymorphism (SNP) typing are believed to be the next tool to improve the analysis of low amounts of DNA in forensic samples[59,60]. However, these process are time-consuming, are not currently cost-effective and no statistical or probabilistic modeling techniques yet exist to maximize the contributor-specific information [60]. In this project, we developed a new pre-DNA-profiling method. Our findings suggest that testosterone and DHT antibodies can be used to screen epithelial cell populations, thereby, distinguishing single source samples from mixtures and possibly predicting DNA yield before DNA analysis in a non-destructive manner. This has the potential for a powerful impact to forensic science. By estimating the quality and quantity of DNA in a touch sample, mixture, low template/trace DNA samples may be more effectively analyzed. Or perhaps, it may be determined that given the mixture complexity and lack of measurable anti-testosterone/DHT staining, there is little DNA and it would not be fruitful to pursue DNA profiling of the sample at this time.

In summary, although there were some limitations to the detection of the number of contributors to a mixed touch DNA sample and more research needs to be explored for this project, our results still demonstrated that testosterone and DHT antibodies and the CK antibody have the potential to be applied for pre-DNA touch evidence analysis in the forensic science field.

4.5 Future Directions

This project will continue, with additional samples tested in order to measure the variance between individuals for the DNA quantity prediction. Also, further investigation of the variance

between individuals and the correlation between CK antibody and DNA quantities will be performed. Finally, a greater number of contributors for mixture samples (3- and 4-person mixtures) touched sequentially, will be examined and the trends measured.

Chapter 5. Conclusion

This project presents a novel pre-DNA-profiling method that may be optimized to characterize touch DNA epithelial cell mixtures rapidly in a non-destructive manner. By utilizing cholesterol-based hormones, testosterone and dihydrotestosterone, and cytokeratin protein-specific antibodies in epithelial cells. This helps sample triaging as well as data analysis and interpretation. The DNA from touch evidence may be preserved, while still providing information including distinguishing cell populations, sequential touching, and DNA content.

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Figures and Tables

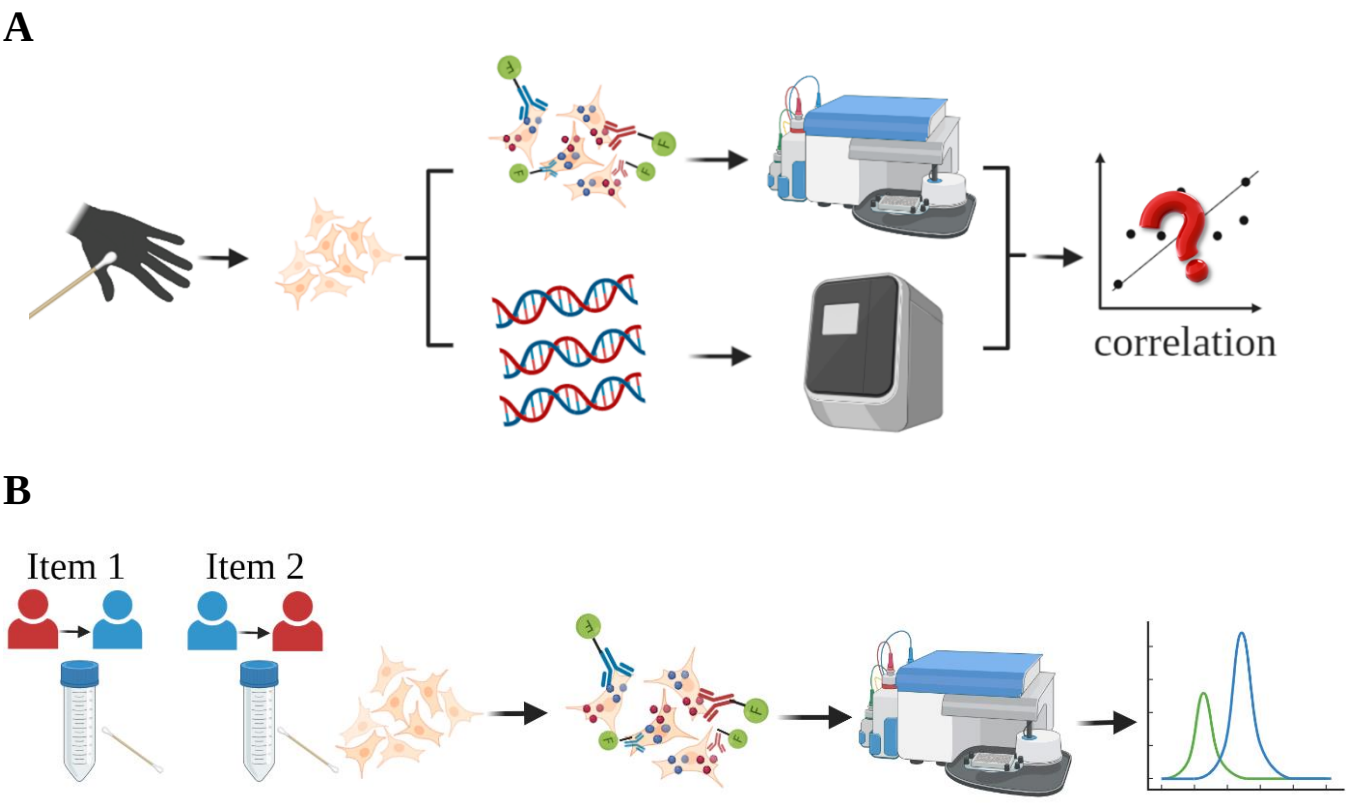
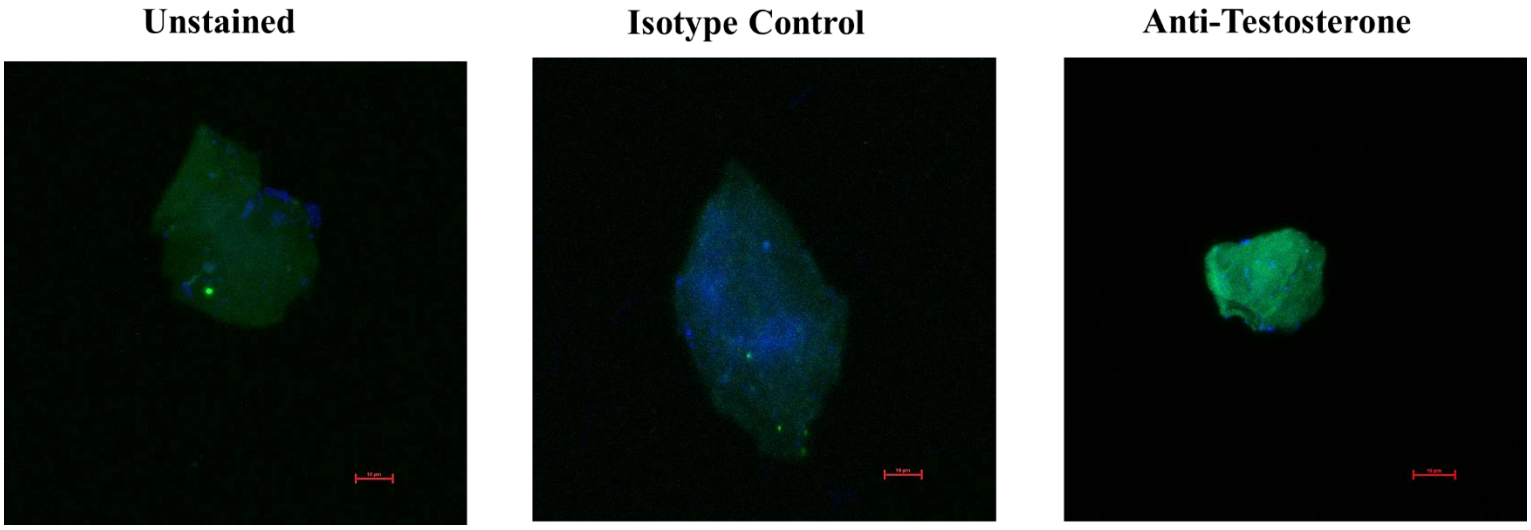


Figure 1. Experimental Design. (**A**) Epithelial cells were collected to analyze DNA quantity by qPCR versus antibody fluorescent signal using flow cytometry. **(B)** Epithelial cells were collected from items to analyze antibody staining patterns in order to test the effect of sequential contact.

A



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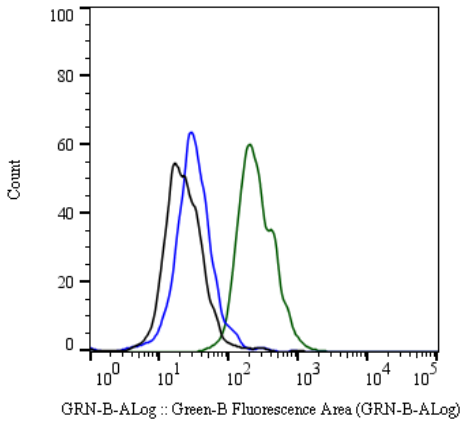

	Sample Name	Count	Mean : GRN-B-ALog
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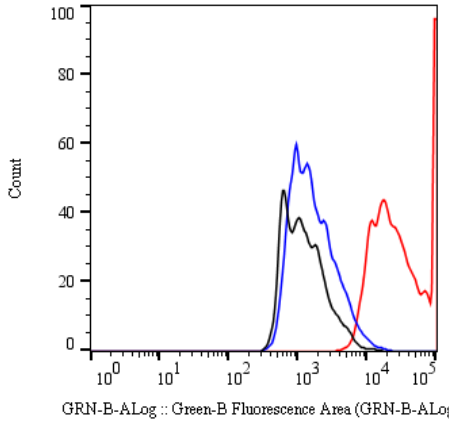
☐	2021-06-07 N27- iso.fcs	1936	41.8
☐	2021-06-07 N27-ab.fcs	1910	296

Figure 2. Testosterone and DHT antibodies have high specificity to their targets in epithelial cells. (A) Representative fluorescence intensity after immunofluorescence (IF) staining of epithelial cells. DNA in epithelial cells were stained with DAPI (blue), and counter-stained with a non-specific isotype control or testosterone antibody (green). **(B)** Histograms of staining patterns detected by flow cytometry. Epithelial cells were unstained (black), isotype control antibody

stained (blue) or testosterone and DHT antibodies (dark green). Optimal 1.85 μg quantities of isotype antibody, 1.85 μg quantities of testosterone and 4.1 μg of DHT antibodies were utilized for staining.

A



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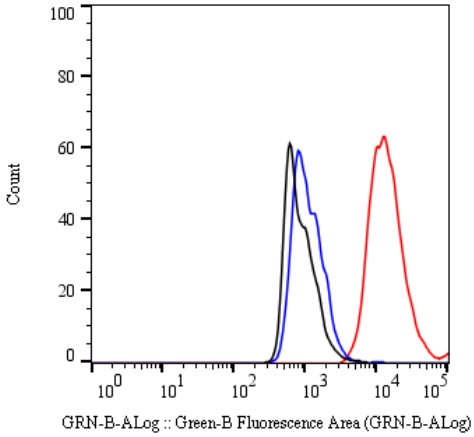
B



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C

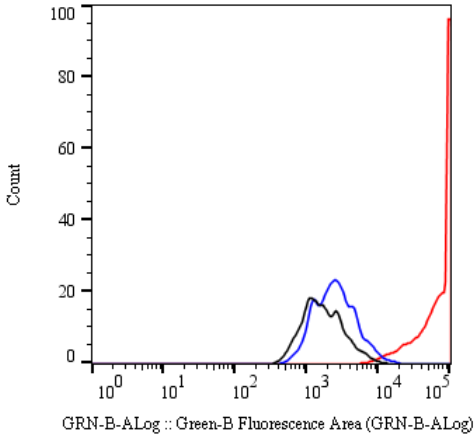

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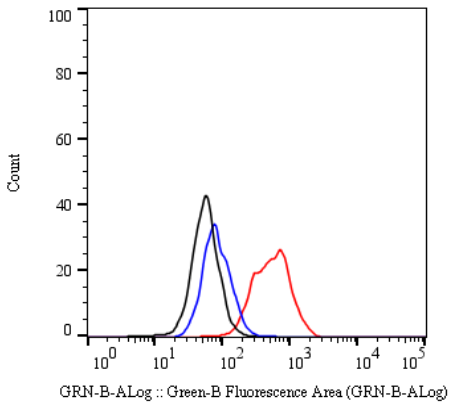
Figure 3. Testosterone and DHT antibodies distinguish 2 individuals in a 2-person mixture sample. Optimal 1.85 µg of testosterone and 4.1 µg of DHT antibodies staining conditions were used for all samples. **(A)** A 1:1 2-person mixture sample (I66 and N27). **(B)** A single source of contributor #1 (N27). **(C) A** single source of contributor #2 (I66). Unstained cells (black), isotype antibody staining cells (blue), and optimized concentrations of testosterone and DHT antibodies

staining cells (red). Note: fluorescent signal of I66 is off-scale so the entire histogram profile cannot be seen.

A



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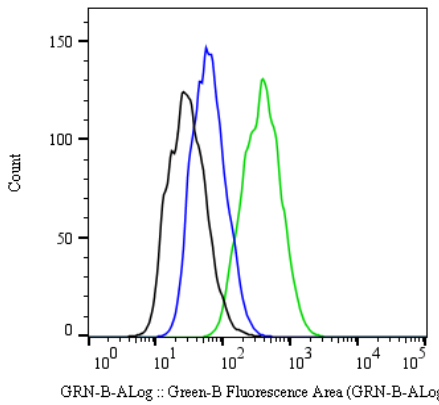

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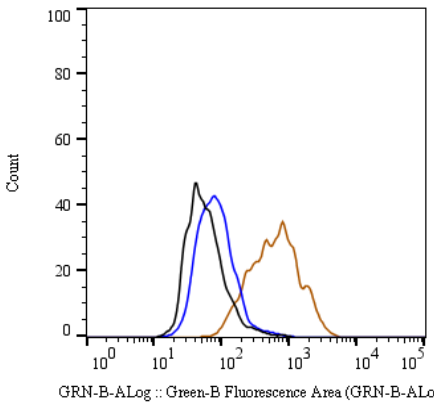
C



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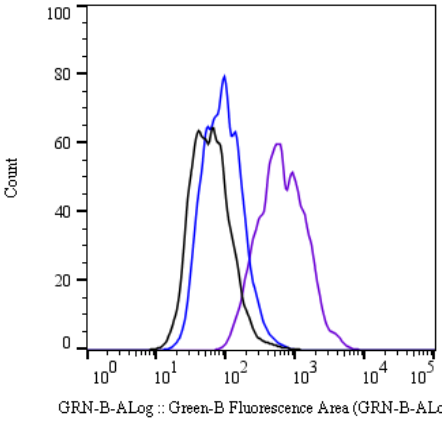
D



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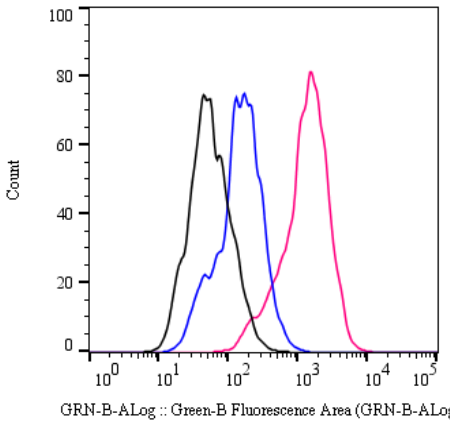
E



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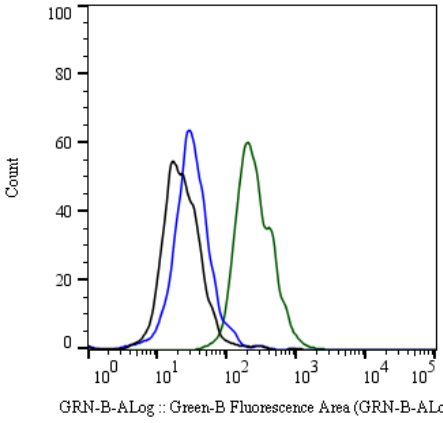

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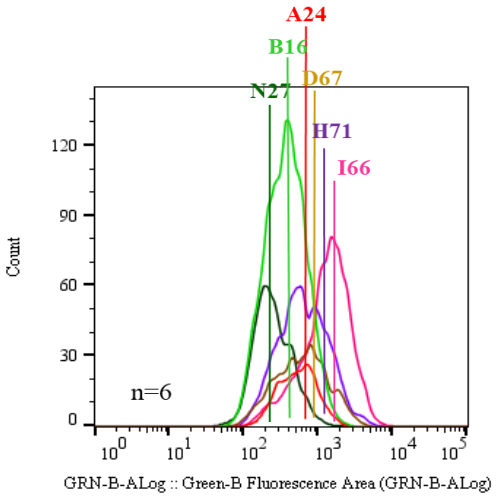
☐	2021-06-07 N27-ab.fcs	1910	296

G



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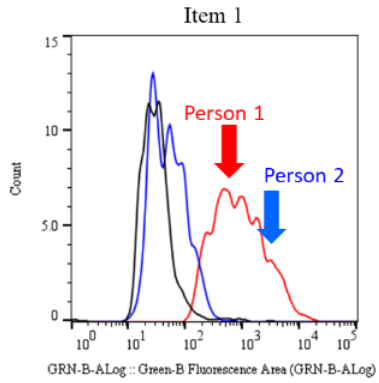
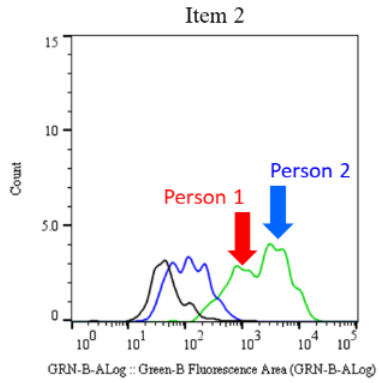
☐	2021-06-08 D67-ab.fcs	1599	805
☐	2021-06-07 H71-ab.fcs	2676	846
☐	2021-06-07 I66-ab.fcs	2963	1584
☐	2021-06-07 N27-ab.fcs	1910	296

Figure 4. Testosterone and DHT antibodies generate personalized fluorescent signal. (A) Optimal quantities of testosterone (1.85 µg) and DHT (4.1 µg) antibodies were used to stain a single source sample - A24 (red). **(B)** Sample B16 (light green). **(C)** H71 (purple). **(E)** I66 (pink). **(F)** N27 (dark green). Unstained cells are shown in black and isotype antibody staining cells in blue. **(G)** Overlay of testosterone and DHT antibodies staining fluorescent histograms for all six single source samples.

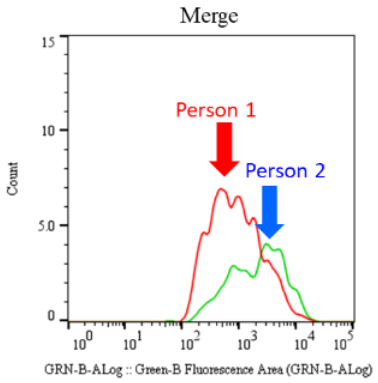
A



Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 order switch I66N27-unstain.fcs	366	53.0
2021-06-02 order switch I66N27-iso.fcs	465	64.2
2021-06-02 order switch I66N27-ab.fcs	432	1456

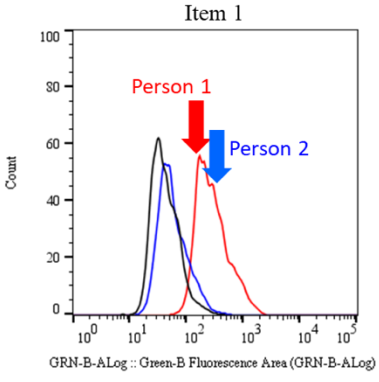


Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 order switch N27I66-unstain.fcs	106	83.4
2021-06-02 order switch N27I66-iso.fcs	166	149
2021-06-02 order switch N27I66-ab.fcs	245	3142

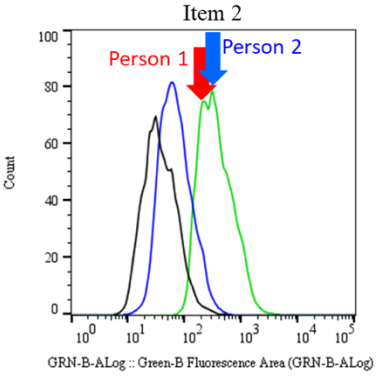


Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 order switch I66N27-ab.fcs	432	1456
2021-06-02 order switch N27I66-ab.fcs	245	3142

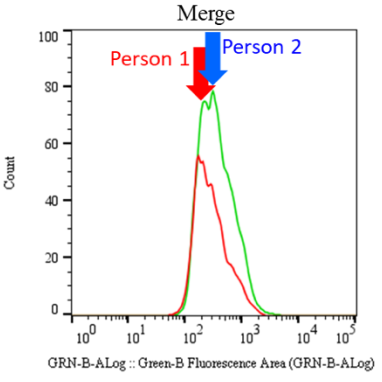
B



Sample Name	Count	Mean : GRN-B-ALog
2021-06-04 order switch B16N27-unstain.fcs	1766	51.1
2021-06-04 order switch B16N27-iso.fcs	1550	71.4
2021-06-04 order switch B16N27-ab.fcs	1821	354



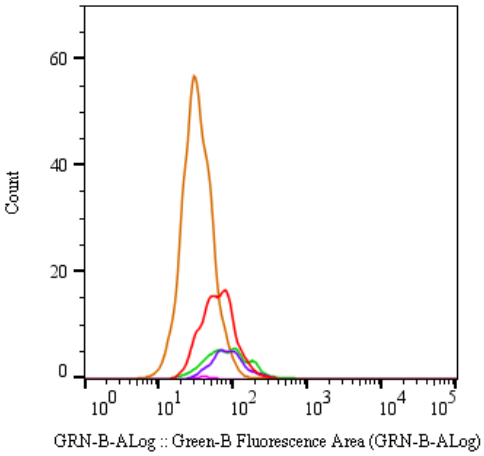
Sample Name	Count	Mean : GRN-B-ALog
2021-06-04 order switch N27B16-unstain.fcs	2601	47.9
2021-06-04 order switch N27B16-iso.fcs	3000	86.2
2021-06-04 order switch N27B16-ab.fcs	3000	447



Sample Name	Count	Mean : GRN-B-ALog
2021-06-04 order switch B16N27-ab.fcs	1821	354
2021-06-04 order switch N27B16-ab.fcs	3000	447

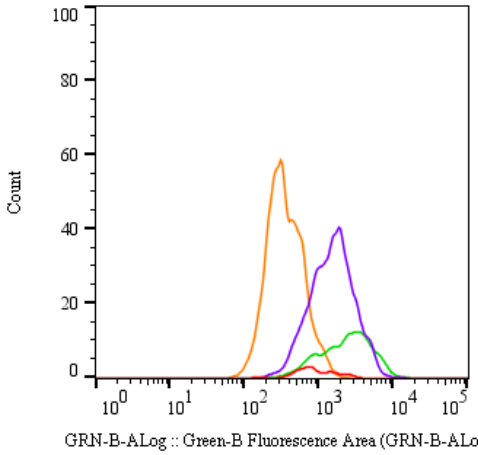
Figure 5. Testosterone and DHT antibodies used to estimate the number of contributors and sequential touching in a 2-person mixture sample. (A) I66 and N27 sequential touching Item #1; N27 and I66 sequential touching Item #2; Overlaid fluorescent histogram (Item #1-red; Item #2-green). **(B)** B16 and N27 sequential touching Item #1; N27 and B16 sequential touching Item #2; Overlaid fluorescent histogram (Item #1-red; Item #2-green). Unstained cells (black), isotype antibody staining cells (blue), optimal 1.85 µg of testosterone and 4.1 µg of DHT antibodies staining cells.

A



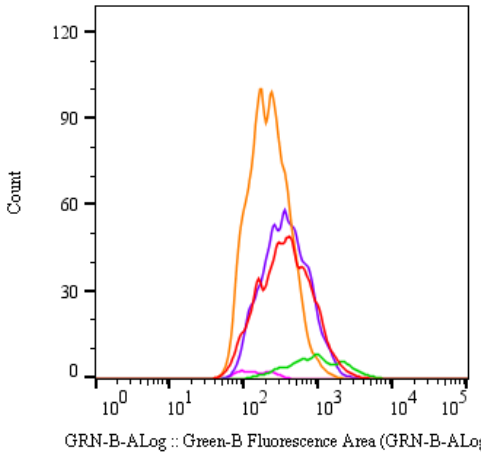
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-11-11 E44-ab.fcs	521	70.5
☐	2021-11-11 I66-ab.fcs	170	99.2
☐	2021-11-11 Jo-ab.fcs	239	112
☐	2021-11-11 N27-ab.fcs	1436	41.7
☐	2021-11-11 SR-ab.fcs	12.0	53.8

B



	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-30 D67-ab.fcs	122	1127
☐	2021-07-30 E44-ab.fcs	1505	1874
☐	2021-07-30 I66-ab.fcs	584	3025
☐	2021-07-30 N27-ab.fcs	1787	419

C



	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-12-07 E44-ab.fcs	2334	494
☐	2021-12-07 I66-ab.fcs	418	1393
☐	2021-12-07 N27-ab.fcs	3711	259
☐	2021-12-07_ Z34-ab.fcs	2432	460
☐	2021-12-07 BC-ab.fcs	111	213
☐	2021-12-07 BC2-ab.fcs	17.0	2165

D

	DNA con.
E44	0.0085
I66	0.0072
Jo	0.0032
N27	0.0116
SR	0.0005

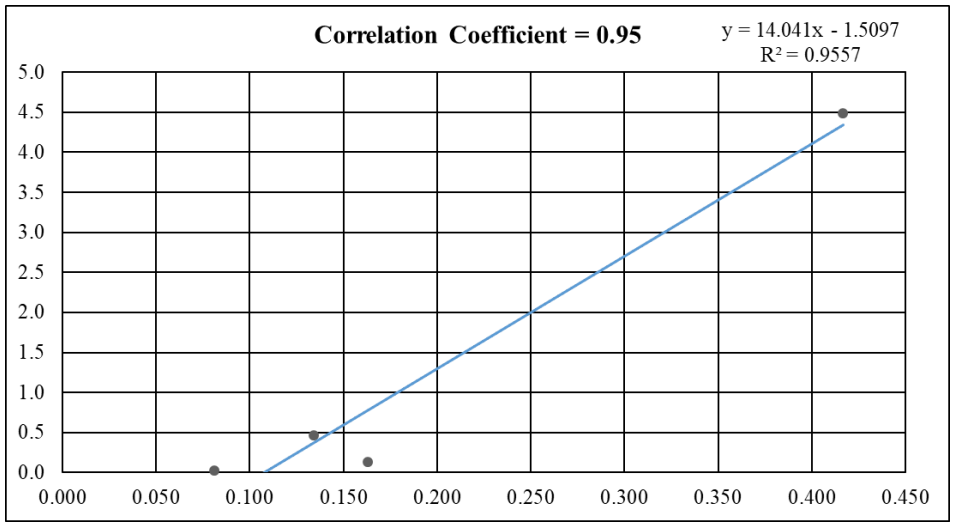
E

	DNA con.
I66	0.191
N27	0.1239
E44	0.0967
D67	0.0825

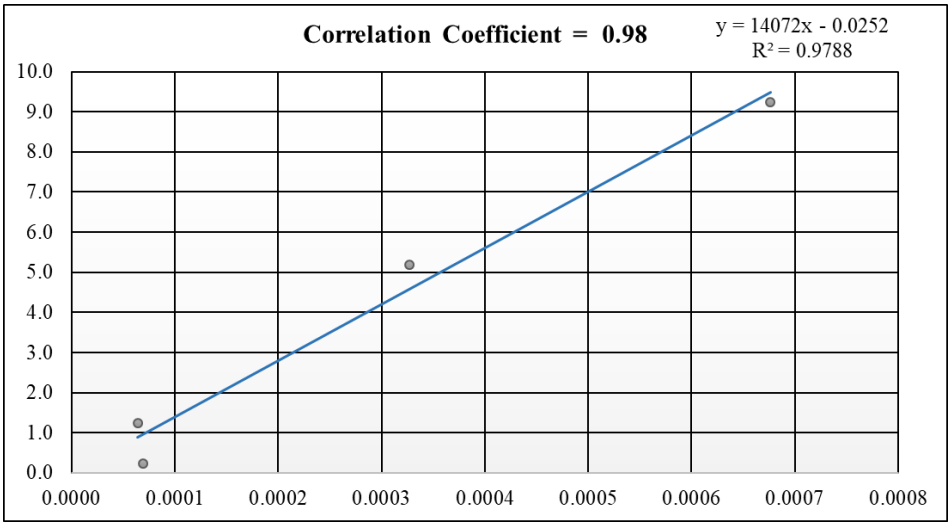
F

	DNA con.
E44	0.0258
I66	0.0024
N27	0.0009
Z34	0.0042
BC	0.0024
BC2	0.0026

G



H



I

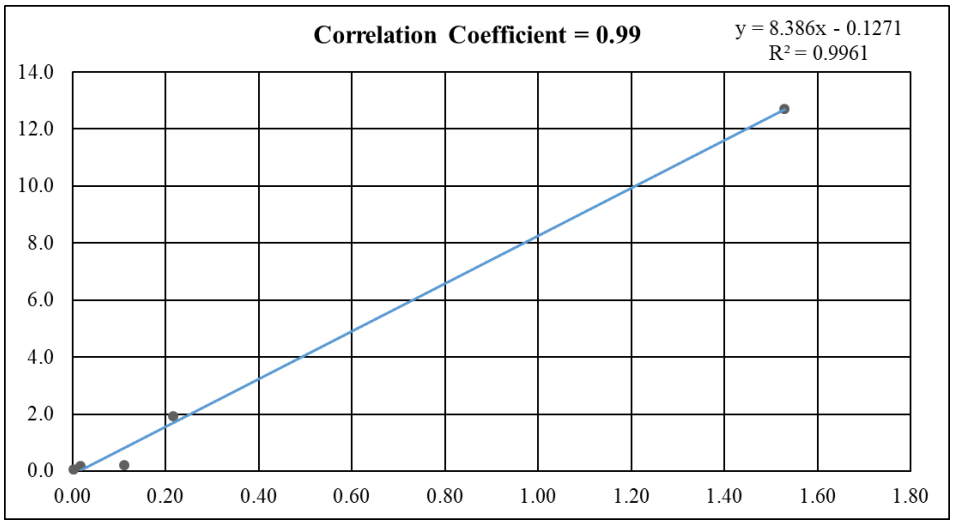
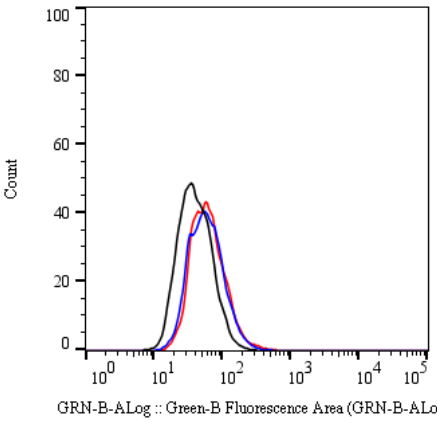


Figure 6. Strong correlations between DNA quantity and the shift in fluorescence in the histograms. (A) Overlaid testosterone staining fluorescent signals for experiment 1. **(B)** Overlaid testosterone and DHT antibody staining fluorescent signals for experiment 2 (panel A). **(C)** Overlaid testosterone and DHT antibodies staining fluorescent signals for experiment 3. Optimal 1.85 µg of testosterone (and) 4.1 µg of DHT antibodies staining in single source samples. **(D)** DNA

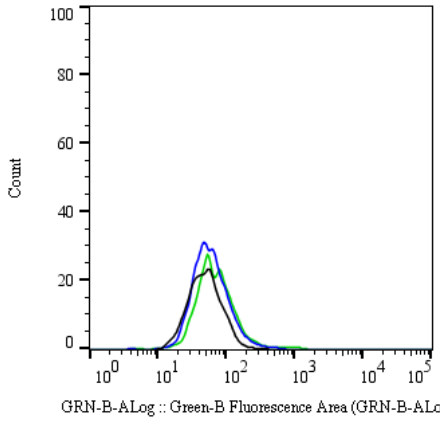
concentration chart for contributors shown in panel A, **(E)** DNA concentration chart for contributors shown in panel B. **(F)** DNA concentration chart for contributors shown in panel C. **(G)** Correlation coefficient (R^2) is calculated for normalized DNA (X-axis) to normalized fluorescent shift (Y-axis) for samples shown in panel A. **(H)** Correlation coefficient (R^2) is calculated for normalized DNA (X-axis) to normalized fluorescent shift (Y-axis) for samples shown in panel B. I66 data point removed as an outlier for linear regression and R^2 analyses. **(I)** Correlation coefficient (R^2) is calculated for normalized DNA (X-axis) to normalized fluorescent shift (Y-axis) for samples shown in panel C. I66 data point removed as an outlier for linear regression and R^2 analyses. DNA was eluted in 30 μL of TE^{-4} buffer for each donor for all experiments shown.

A



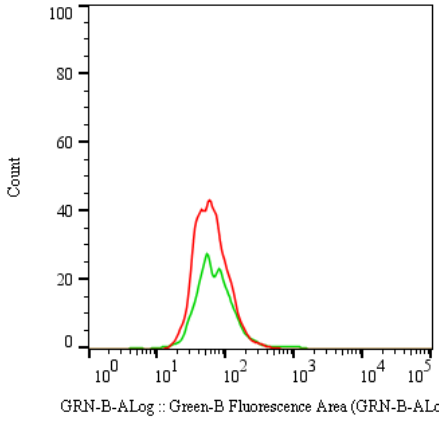
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-21 B16-unstain.fcs	1631	49.7
☐	2021-07-21 B16-iso.fcs	1402	71.7
☐	2021-07-21 B16-ab.fcs	1401	87.6

B



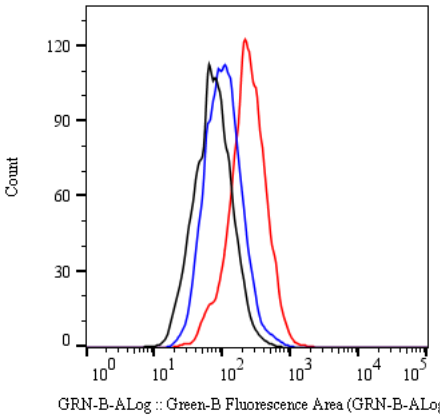
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-21 D67-unstain.fcs	781	62.7
☐	2021-07-21 D67-iso.fcs	1034	76.9
☐	2021-07-21 D67-ab.fcs	898	125

C



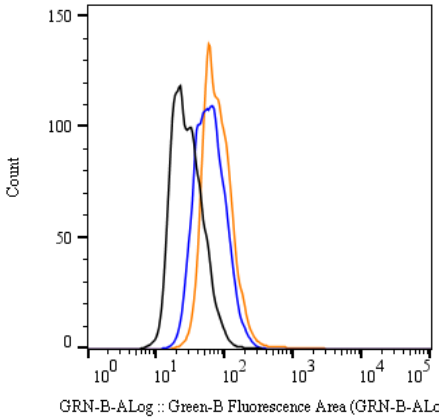
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-21 B16-ab.fcs	1401	87.6
☐	2021-07-21 D67-ab.fcs	898	125

D



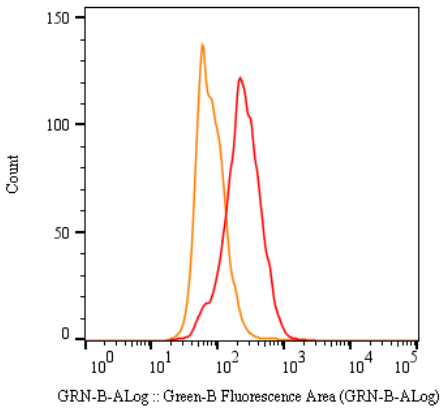
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-29 B16-unstain.fcs	3875	87.5
☐	2021-07-29 B16-iso.fcs	4024	124
☐	2021-07-29 B16-ab.fcs	3890	273

E



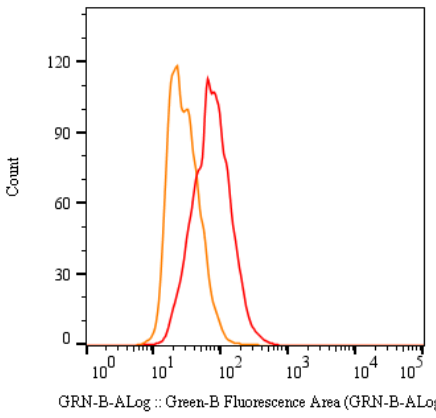
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-29 N27-unstain.fcs	3464	33.7
☐	2021-07-29 N27-iso.fcs	3437	70.4
☐	2021-07-29 N27-ab.fcs	3437	109

F



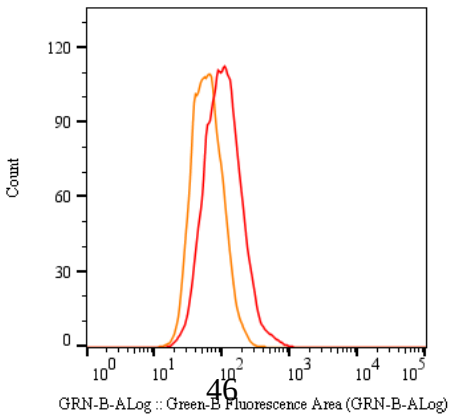
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-29 B16-ab.fcs	3890	273
☐	2021-07-29 N27-ab.fcs	3437	109

G



	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-29 B16-unstain.fcs	3875	87.5
☐	2021-07-29 N27-unstain.fcs	3464	33.7

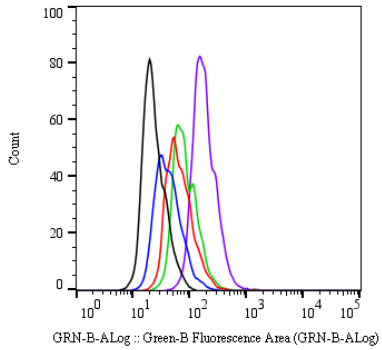
H



	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-29 B16-iso.fcs	4024	124
☐	2021-07-29 N27-iso.fcs	3437	70.4

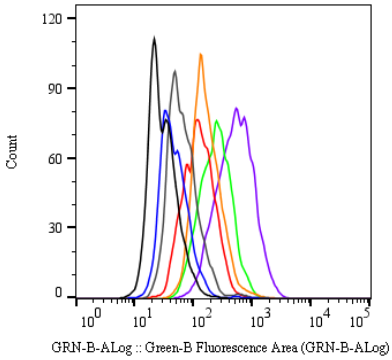
Figure 7. AE1 CK antibody has low specificity to CK in epithelial cells. (A) Single source sample - B16 (red). **(B)** Single source sample D67 (light green). **(C)** Overlaid AE1 CK antibody staining fluorescent signals (B16 and D67). **(D)** Single source sample - B16 (red). **(E)** Single source sample - N27 (orange). **(F)** Overlaid AE1 CK antibody staining fluorescent signals (B16 and N27). Unstained cells (black), isotype antibody staining cells (blue) **(G)** Overlaid unstained cells fluorescent signals (B16 and N27). **(H)** Overlaid isotype antibody staining fluorescent signals (B16 and N27). Optimal 2.225 μg of AE1 CK antibody staining was used for samples in panels A and B. Optimal 4.45 μg of AE1 CK antibody staining was used for samples in panels D and E.

A



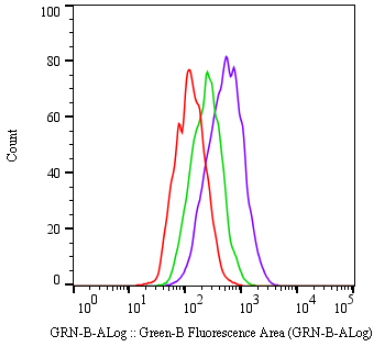
Sample Name	Count	Mean : GRN-B-ALog
2021-08-18 unstain.fcs	1805	26.9
2021-08-18 iso.fcs	1399	48.9
2021-08-18 2.5uL.fcs	1494	88.3
2021-08-18 5uL.fcs	1484	101
2021-08-18 10uL.fcs	2014	202

B



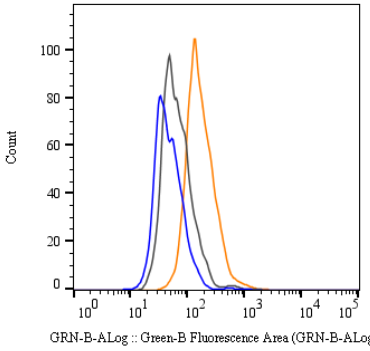
Sample Name	Count	Mean : GRN-B-ALog
2021-09-17 N27 unstain.fcs	2840	36.7
2021-09-17 N27 iso-2.5.fcs	2291	65.0
2021-09-17 N27 iso-5.fcs	2828	91.0
2021-09-17 N27 iso-10.fcs	2794	209
2021-09-17 N27 ab-2.5.fcs	2636	146
2021-09-17 N27 ab-5.fcs	2617	276
2021-09-17 N27 ab-10.fcs	3338	628

C



Sample Name	Count	Mean : GRN-B-ALog
2021-09-17 N27 ab-2.5.fcs	2636	146
2021-09-17 N27 ab-5.fcs	2617	276
2021-09-17 N27 ab-10.fcs	3338	628

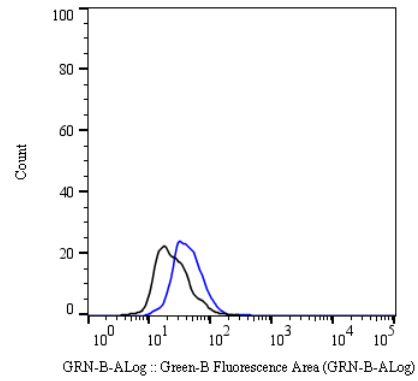
D



Sample Name	Count	Mean : GRN-B-ALog
2021-09-17 N27 iso-2.5.fcs	2291	65.0
2021-09-17 N27 iso-5.fcs	2828	91.0
2021-09-17 N27 iso-10.fcs	2794	209

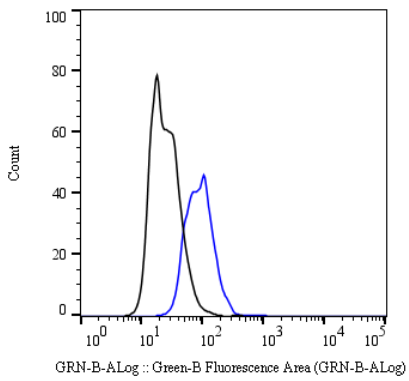
Figure 8. Non-specific isotype antibody signal increased with the increase of antibody quantity. (A) concentration test of AE1 CK antibody staining fluorescent signals shown for experiment #1. **(B)** concentration test of AE1 CK antibody staining fluorescent signals for experiment #2. Unstained cells (black), isotype antibody staining cells (blue), AE1 CK antibody staining cells (colors) **(C)** Overlaid AE1 CK antibody staining fluorescent signals. **(D)** Overlaid isotype antibody staining fluorescent signals. 2 = 2.225 μg, 5 = 4.45 μg, 10 = 8.9 μg of AE1 CK or isotype antibody.

A



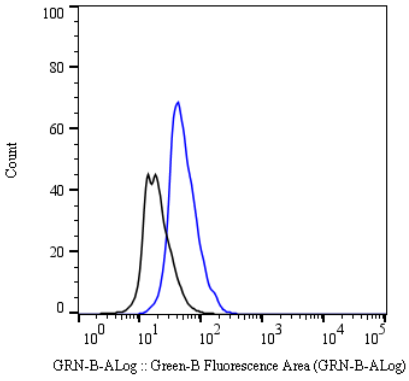
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 N27-adj-unstain.fcs	797	35.6
2021-06-02 N27-adj-iso.fcs	765	50.8

B



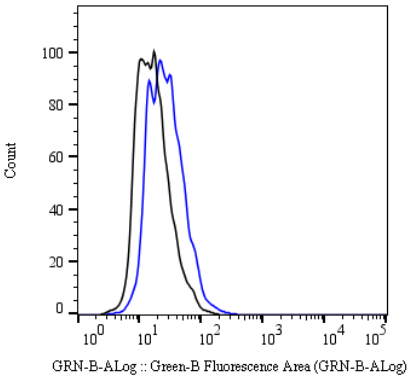
Sample Name	Count	Mean : GRN-B-ALog
2021-07-28 N27-old-unstain.fcs	2216	30.2
2021-07-28 N27-old-iso.fcs	1355	104

C



Sample Name	Count	Mean : GRN-B-ALog
2021-07-30 N27-unstain.fcs	1229	33.1
2021-07-30 N27-iso.fcs	1786	60.6

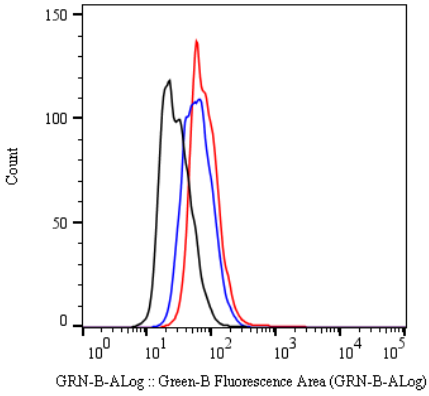
D



Sample Name	Count	Mean : GRN-B-ALog
2021-10-28 N27-unstain.fcs	3465	20.9
2021-10-28 N27-iso.fcs	3623	33.7

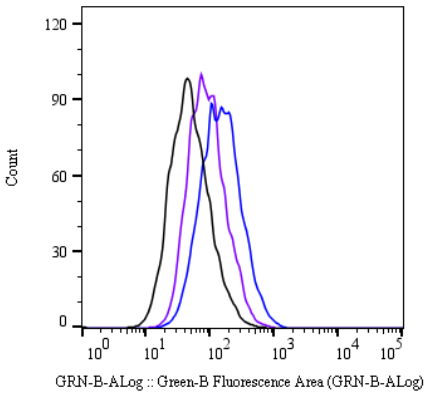
Figure 9. Non-specific binding of isotype antibody decreased when cells were blocked with 2% BSA. Optimal 1.85 ug of isotype antibody was applied to all samples tested. **(A)** Single source sample with commercial blocking buffer for experiment #1. **(B)** Single source sample with commercial blocking buffer for experiment #2. **(C)** Single source sample with commercial blocking buffer for experiment #3. (**D**) Single source sample with 2% BSA blocking buffer. Unstained cells (black), isotype antibody staining cells (blue).

A



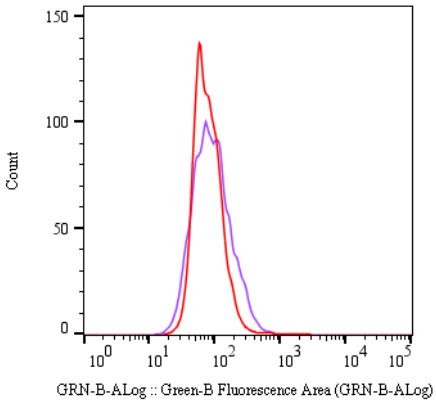
Sample Name	Count	Mean : GRN-B-ALog
2021-07-29 N27-unstain.fcs	3464	33.7
2021-07-29 N27-iso.fcs	3437	70.4
2021-07-29 N27-ab.fcs	3437	109

B



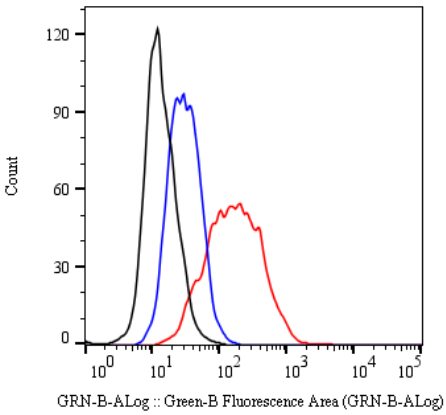
Sample Name	Count	Mean : GRN-B-ALog
2021-10-01 Z34 unstain.fcs	3721	63.6
2021-10-01 Z34 iso.fcs	3735	185
2021-10-01 Z34 ab.fcs	3727	112

C



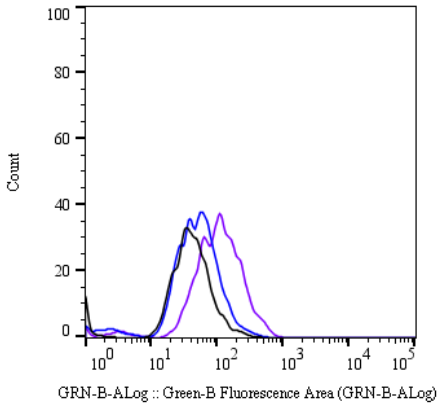
Sample Name	Count	Mean : GRN-B-ALog
2021-07-29 N27-ab.fcs	3437	109
2021-10-01 Z34 ab.fcs	3727	112

D



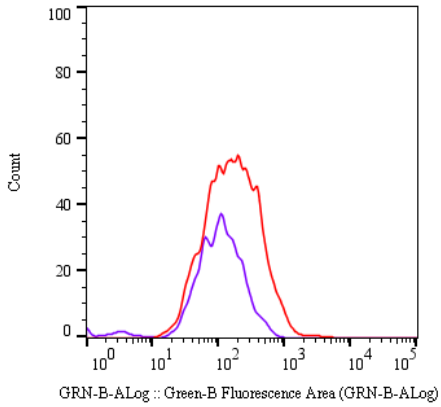
Sample Name	Count	Mean : GRN-B-ALog
2021-10-26 N27-unstain.fcs	3331	15.5
2021-10-26 N27-iso.fcs	3230	34.6
2021-10-26 N27-ab.fcs	3144	251

E



Sample Name	Count	Mean : GRN-B-ALog
2021-10-26 Z34-unstain.fcs	1198	50.8
2021-10-26 Z34-iso.fcs	1579	62.9
2021-10-26 Z34-ab.fcs	1588	136

F



Sample Name	Count	Mean : GRN-B-ALog
2021-10-26 N27-ab.fcs	3144	251
2021-10-26 Z34-ab.fcs	1588	136

Figure 10. AE1/AE3 CK antibody has high specificity to CK in epithelial cells. (A) Optimal 2.225 ug of AE1 CK antibody staining in a single source sample - N27 (red). **(B)** Z34 (purple). **(C)** Overlaid AE1 CK antibody staining fluorescent signals for both single source samples. **(D)** Optimal 2.225 ug of AE1/AE3 CK antibody staining in a single source sample - N27 (red). **(E)**

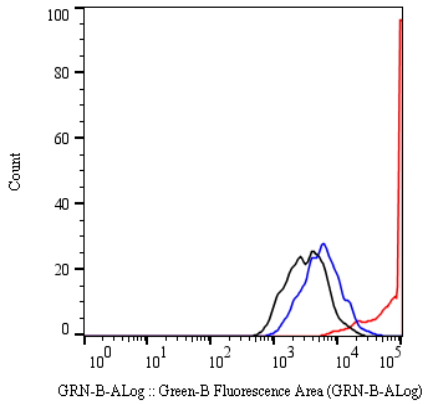
Z34 (purple). **(F)** Overlaid AE1/AE3 CK antibody staining fluorescent signals fluorescent signals for both single source samples. Unstained cells (black), isotype antibody staining cells (blue).

Vita

Christin Lee was born in Iowa and raised in Taiwan. She is currently a second-year graduate student in the Forensic Science-Biology track at Virginia Commonwealth University. She received her Bachelor of Science degree in Biomedical Sciences from Chung Shan Medical University, Taiwan in 2018, and received her Master of Science degree in Clinical Medicine from National Cheng Kung University, Taiwan in 2020. She worked as a research assistant and performed her thesis research which is focusing on investigating the mechanism of the effect by sodium-glucose cotransporter-2 inhibitor on obesity-associated cardiac injury in Dr. Ping-Yen Liu's Lab. She worked as a student research intern at the Virginia Department of Forensic Science, Forensic Biology Section and has performed a poster presentation at the 2022 AAFS annual meeting.

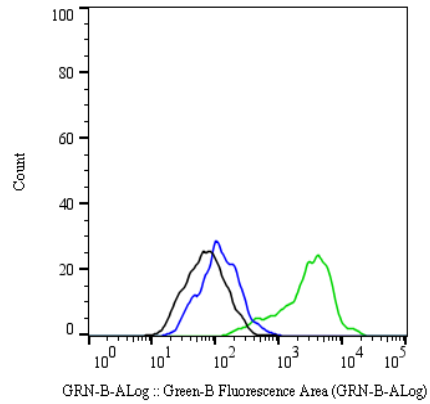
Supplementary

A



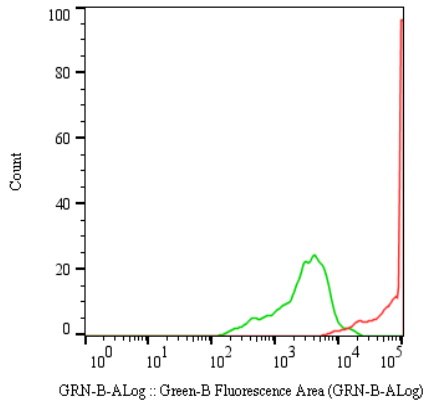
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 I66-ori-unstain.fcs	1142	4136
2021-06-02 I66-ori-iso.fcs	1084	6876
2021-06-02 I66-ori-ab.fcs	1150	82946

B



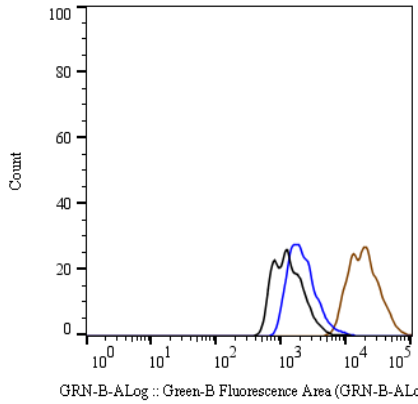
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 I66-adj-unstain.fcs	1134	92.0
2021-06-02 I66-adj-iso.fcs	1105	150
2021-06-02 I66-adj-ab.fcs	1074	3542

C



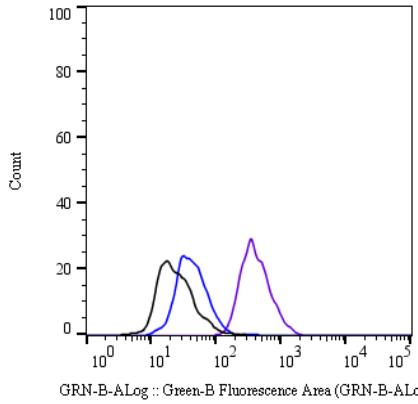
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 I66-ori-ab.fcs	1150	82946
2021-06-02 I66-adj-ab.fcs	1074	3542

D



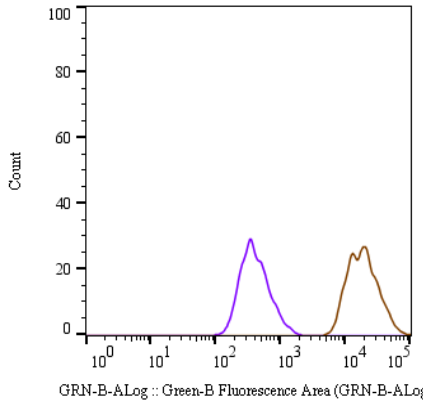
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 N27-ori-unstain.fcs	795	1762
2021-06-02 N27-ori-iso.fcs	779	2477
2021-06-02 N27-ori-ab.fcs	855	20803

E



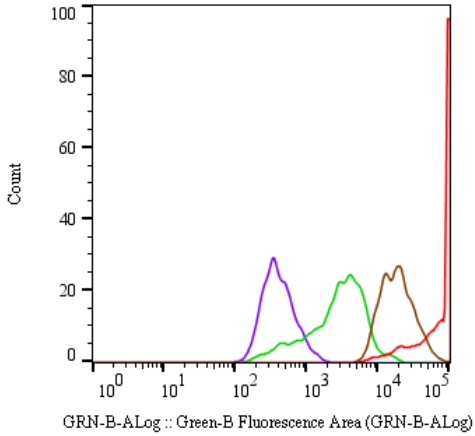
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 N27-adj-unstain.fcs	797	35.6
2021-06-02 N27-adj-iso.fcs	765	50.8
2021-06-02 N27-adj-ab.fcs	819	464

F



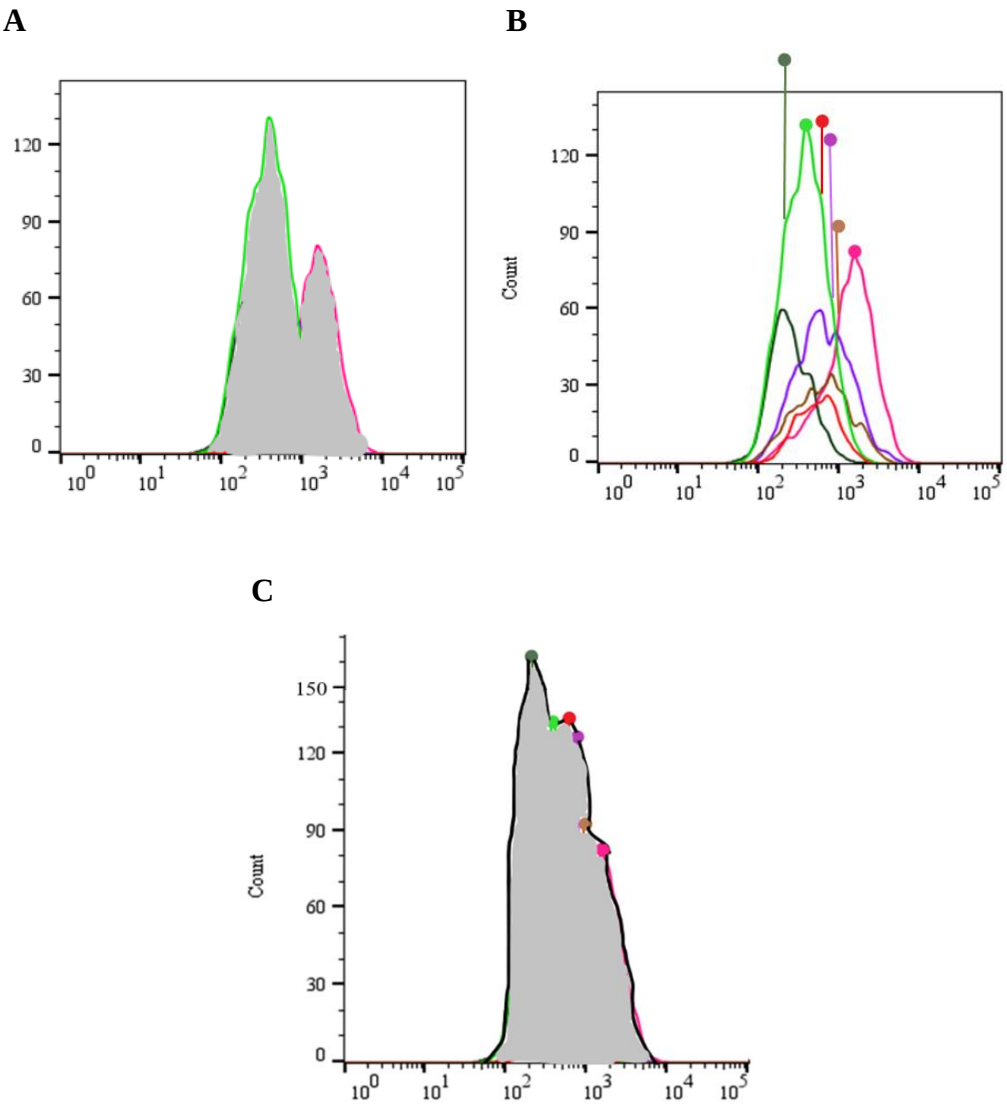
Sample Name	Count	Mean : GRN-B-ALog
2021-06-02 N27-ori-ab.fcs	855	20803
2021-06-02 N27-adj-ab.fcs	819	464

G



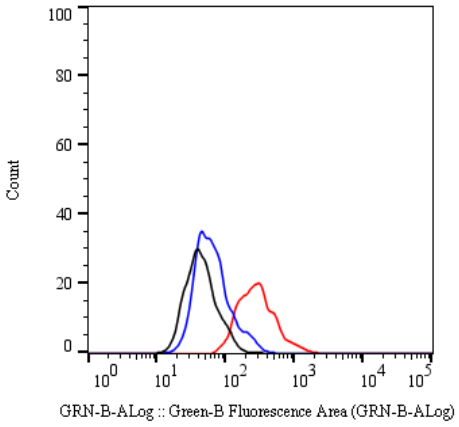
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-06-02 I66-ori-ab.fcs	1150	82946
☐	2021-06-02 N27-ori-ab.fcs	855	20803
☐	2021-06-02 I66-adj-ab.fcs	1074	3542
☐	2021-06-02 N27-adj-ab.fcs	819	464

Supplementary Figure 1. Effect of adjustment of photomultiplier voltages for epithelial cells antibodies stain with testosterone and DHT antibodies. (**A**) Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - I66 with high voltage. (**B**) Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - I66 with low voltage. (**C**) Histogram overlays of epithelial cells stained with testosterone and DHT antibodies (I66). (**D**) Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - N27 with high voltage. (**E**) Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - N27 with low voltage. (**F**) Histogram overlays of epithelial cells stained with testosterone and DHT antibodies (N27). Unstained cells (black), isotype antibody staining cells (blue) testosterone and DHT antibodies staining cells (colors). (**G**) Overlaid testosterone and DHT antibodies staining fluorescent signals.



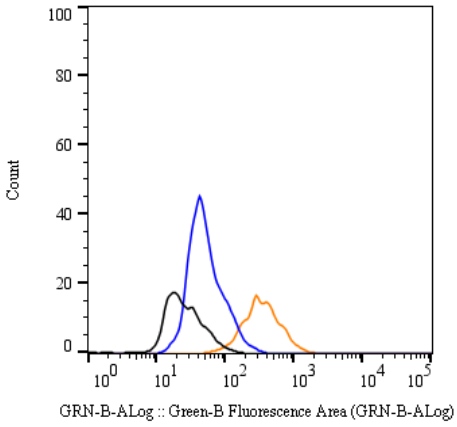
Supplementary Figure 2. Overlaid single source fluorescent histogram. (A) Overlapping six single source patterns from Figure 4 panel G. **(B)** Mathematical modeling for each peak from Figure 4 panel G. **(C)** Overlapping six single source patterns from Supplementary Figure 2 panel B when using the add-effect mathematical modeling method.

A



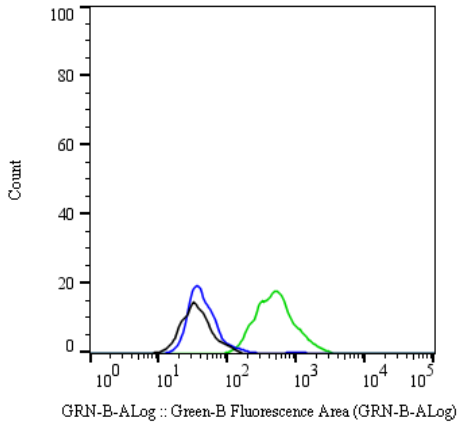
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-08 E44-unstain.fcs	874	54.7
☐	2021-07-08 E44-iso.fcs	1092	78.9
☐	2021-07-08 E44-ab.fcs	703	349

B



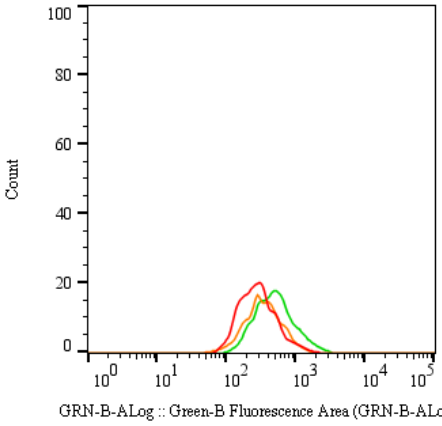
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-08 N27-unstain.fcs	603	32.2
☐	2021-07-08 N27-iso.fcs	1242	65.7
☐	2021-07-08 N27-ab.fcs	555	407

C



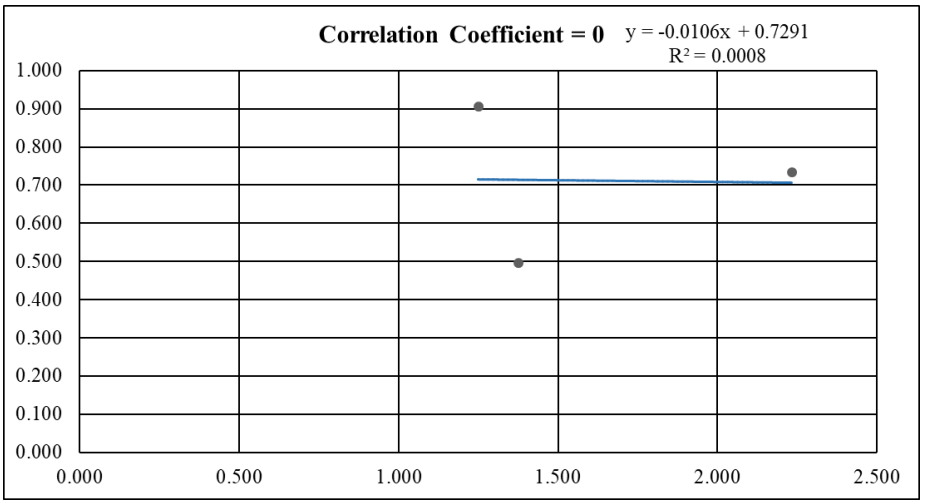
	Sample Name	Count	Mean : GRN-B-ALog
☐	2021-07-08 Z34-unstain.fcs	410	41.0
☐	2021-07-08 Z34-iso.fcs	510	107
☐	2021-07-08 Z34-ab.fcs	660	598

D



	Sample Name	Count	Mean : GRN-B-ALog	DNA con.
☐	2021-07-08 E44-ab.fcs	703	349	**0.654**
☐	2021-07-08 N27-ab.fcs	555	407	**0.0477**
☐	2021-07-08 Z34-ab.fcs	660	598	**0.0202**

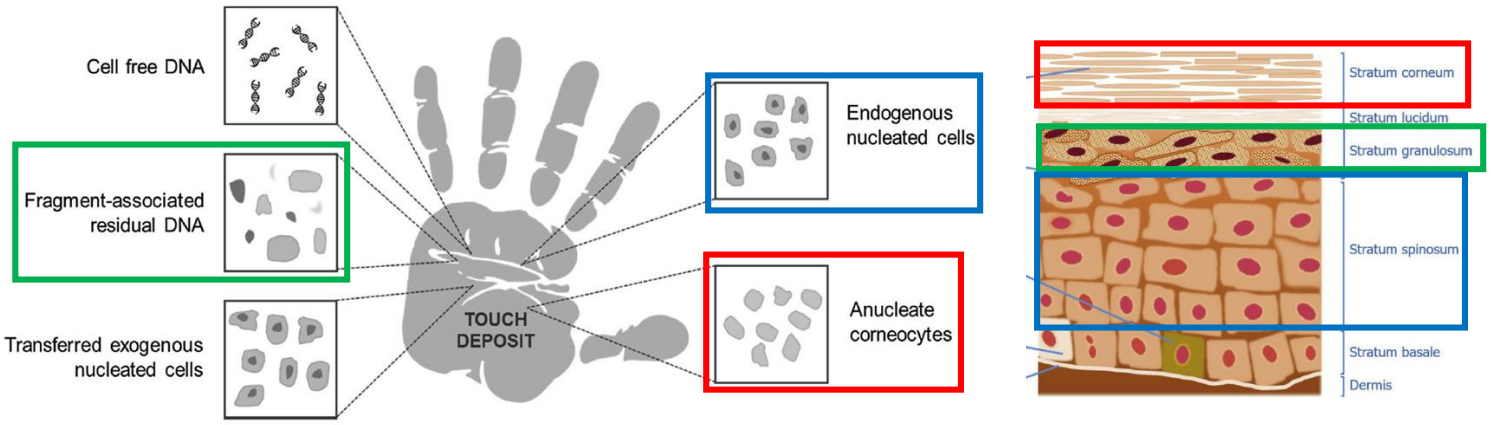
E



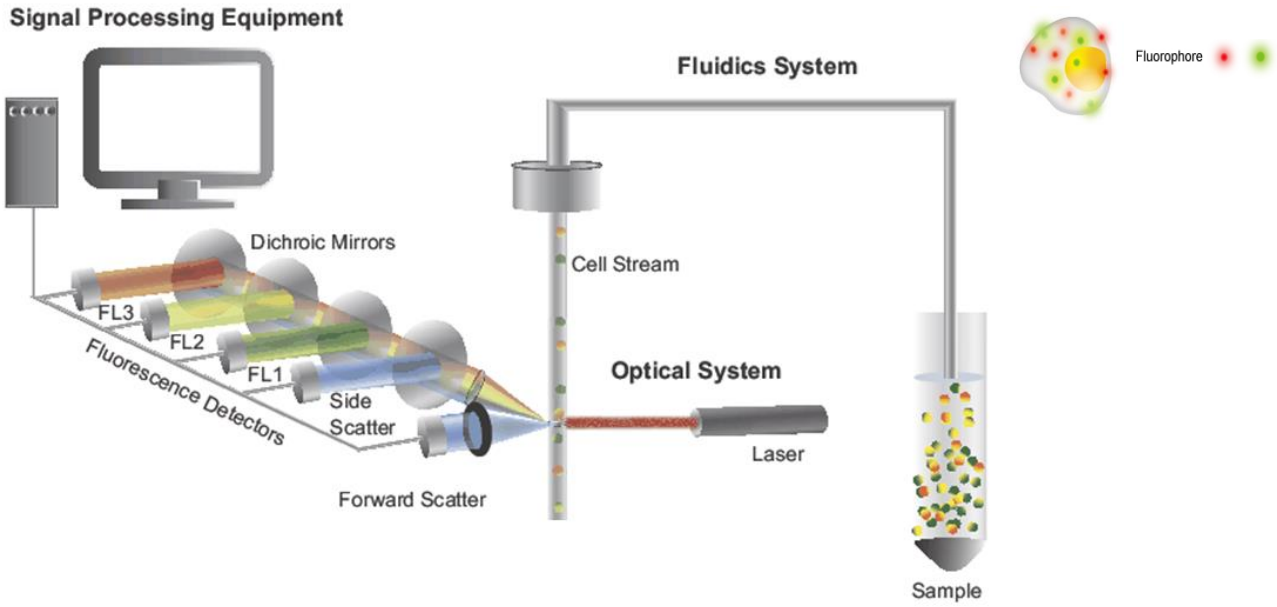
Supplementary Figure 3. Cell-free DNA need to be removed. (A) Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - E44 (red). **(B)** Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a single source sample - N27 (orange). **(C)** Optimal 1.85 ug of testosterone and 4.1 ug of DHT antibodies staining in a

single source sample - Z44 (green). **(D)** Overlaid testosterone and DHT antibodies staining fluorescent signals. Unstained cells (black), isotype antibody staining cells (blue). **(E)** Correlation coefficient (R^2) is calculated for normalized DNA (X-axis) to normalized fluorescent shift (Y-axis).

Appendix



Appendix Figure 1. Potential sources of touch DNA[5]. Touch DNA from skin cells is primarily from keratinocytes, nucleated epithelial cells, residual cell fragments, and cell free DNA



Appendix Figure 2. Flow cytometry fundamental principle[45]**.** Forward Scatter light measures the size of the cell. Side Scatter light measures the internal complexity of the cell. Fluorescently conjugated antibodies in the cells may emit fluorescence, these signals are analyzed by the multicolor-detector.