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EQUUS CABALLUS SEMEN USED AS A CARRIER TO INCREASE HUMAN DNA FROM SIMULATED SEXUAL ASSAULT SWABS FOR FORENSIC SCIENCE APPLICATIONS

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EQUUS CABALLUS SEMEN USED AS A CARRIER TO INCREASE HUMAN DNA FROM SIMULATED SEXUAL ASSAULT SWABS FOR FORENSIC SCIENCE APPLICATIONS

A THESIS APPROVED FOR THE
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Abstract

Currently, there are approximately 200,000 untested sexual assault examination kits in the United States. One reason there are so many untested kits is due to the misconceptions associated with DNA analysis. Since its creation, the Sexual Assault Kit Initiative has reported that 97,000 kits have been tested to completion. While grants are helpful for decreasing the backlog, there are instances where funding has run out or been cut, allowing for a resurgence in the backlog. Therefore, research and development are necessary to reduce the sexual assault examination kit backlog. Conventional differential extraction remains the primary method for separating sperm and epithelial cells despite causing a 94%-98% loss of sperm cells. Despite nearly forty years of innovations, there are no methodologies that improve the yield of male DNA without sperm cell loss. Recent advancements in technology involving carrier RNA have been used to dramatically increase the male DNA recovery without sacrificing sperm cell yield. Therefore, this research investigated the use of a non-human semen sample (horse) as a carrier method to determine if a non-human carrier DNA could be used in place of RNA. It was hypothesized that the carrier DNA should act as a protective barrier during the washing steps of differential extractions reducing sperm cell loss. The thesis studied simulated sexual assault samples in triplicate. The samples were extracted using differential and organic extractions. Finally, the DNA was quantified using Quantifiler™ HP kit to determine the overall human DNA yield. Despite the addition of non-human semen, the DNA concentrations of the experimental group were lower than the control. Therefore, the hypothesis was refuted due to decreased human DNA with the samples containing horse semen. Future research should focus on creating a more tightly packed sperm cell pellet during the centrifugation process.

Literature Review

Development of the Differential Extraction Method

In 1985, researchers from the University of Leicester attempted to show that polymorphic minisatellite loci can be used to help identify suspects involved in sexual assault cases. Up until that time, one way of forensically analyzing semen stains was using five protein markers: phosphoglucomutase, glyoxalase I, α1-3-N-acetylgalactosaminyltransferase, α1-3-galactosyltransferase, Lewis, and peptidase A. Because these genetic markers are present in vaginal secretions, it was incredibly challenging to differentiate between a victim and a suspect to the point that analysts could only trust negative results when comparing a known and an unknown sample. Gill et al. (1985) attempted to determine if high molecular weight DNA could be obtained from forensic samples to utilize minisatellites to aid in the identification of suspects involved in rape cases. They tested bloodstains, semen stains, and vaginal swabs with and without semen. DNA was extracted from samples of whole blood, whole semen, vaginal fluid, hair roots, bloodstains, and semen stains by incubating overnight in a mixture of 0.01M tris(hydroxymethyl)aminomethane hydrochloride, 0.01M ethylenediaminetetraacetic acid, 0.1M sodium chloride, 2% sodium dodecyl sulfate, 20μg/mL proteinase K, and 0.039M dithiothreitol. Following the overnight incubation, the extracted DNA was purified using phenol/chloroform extractions and precipitated using sodium acetate and absolute ethanol. A final washing step with 70% ethanol was performed after pelleting the DNA via centrifugation.

Two groups of researchers verified the results of Gill et al. (1985) four years later. During this time, one prevalent method to analyze samples from sexual assaults was to identify sperm cells, or spermatozoa, in aqueous extracts from evidentiary swabs. Because vaginal cells are present in the evidentiary samples in large volumes, it can be challenging to microscopically

identify sperm cells, especially if they do not retain their tails. Therefore, it became advantageous to separate the two types of cells. Chapman et al. (1989) found that using sodium dodecyl sulfate along with proteinase K digested vaginal cells while leaving the spermatozoa intact. Sperm cells are not lysed in this manner due to the disulfide bonds in the protamines found in the sperm heads (Chapman et al., 1989). Later, it was found that proteinase K also partially digests spermatozoa (Iwasaki et al., 1989). Iwasaki et al. (1989) found that even after only 30 minutes, incubating in proteinase K caused some sperm cells to have deformed heads or be reduced to tails. Despite losing male cells, both Chapman et al. (1989) and Iwasaki et al. (1989) found that using proteinase K was much more efficient at separating the sperm from the female cells, confirming the findings of Gill et al. (1985) (Chapman et al., 1989; Iwasaki et al., 1989). The same issues that plagued Gill et al. (1985) also apply here. A different issue that arose was the pre-mature lysing of sperm cells. This pre-mature lysing contributes to cross-contamination between the fractions.

Physical and Chemical Modifications

Mild Preferential Lysis

Moving out of the 1980s, the first modification to Gill et al.'s (1985) preferential lysis methodology arose from the need to retain as much sperm DNA as possible. As stated previously, some spermatozoa are denatured during the first incubation step with sodium dodecyl sulfate and proteinase K. Sexual assault swabs have a higher concentration of vaginal cells than sperm cells; therefore, it is crucial to retain all of the sperm cells or as close to all as possible. Wiegand et al. (1992) proposed a preferential lysis method in which the male and female cells are not separated to reduce the loss of male DNA. Instead, both types of cells were extracted from the evidentiary swabs using a milder lysis buffer. This lysis buffer contained 0.01M tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), 0.4M sodium chloride, 0.002M

ethylenediaminetetraacetic acid, and 2000μg/mL proteinase K. 20% sodium dodecyl sulfate was added to the previous solution. The main difference between Wiegand et al.'s (1992) digestion solution and Gill et al.'s (1985) solution was the absence of dithiothreitol, which was added in a later step. Following a 40-minute incubation, the extracts were centrifuged to separate the extracts from the swab substrate. After discarding the substrate, the extracts were further centrifuged to pellet the undigested sperm cells. The supernatant was removed and extracted in phenol/chloroform/isoamyl alcohol. In addition to using different concentrations of the reagents described in Gill et al. (1985), the authors did not perform the several washing steps and subsequent DNA transfers to prevent sperm cell loss. The remaining sperm pellet was extracted using 50μL proteinase K, 12μL sodium dodecyl sulfate, and 25μL dithiothreitol and purified using phenol/chloroform/isoamyl alcohol. Further DNA analysis proved that using mild preferential lysis to reduce the number of vaginal cells increased the likelihood of obtaining quality male DNA from mixed samples (Wiegand et al., 1992). This modification towards gentler lysis reduced sperm loss; however, the protocol still utilizes toxic compounds. In addition, it does not solve the time bottleneck, as it took the researchers numerous hours to extract the samples fully.

Two-Step Preferential Lysis

The second modification to Gill et al.'s differential extraction protocol came in 1995 when Yoshida et al. (1995) developed a two-step form of preferential lysis to further ensure the complete separation of sperm cells from vaginal cells. With this modification, the researchers extracted a mixed stain sample by performing two steps using two lysis buffer solutions. The first lysis step used 0.01M tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), 0.01M ethylenediaminetetraacetic acid, 0.1M sodium chloride, 1% sodium dodecyl sulfate, and 100μg/mL proteinase K. In contrast, Gill et al. (1985) used 2% sodium dodecyl sulfate and

20µg/mL proteinase K. Following a 3-hour incubation period, the samples were transferred to new reaction tubes to reduce the chance of contamination from the substrate and undigested cellular components. After centrifugation, the supernatant was removed, leaving the pelleted sperm cells behind. The pellet was washed with 0.1mL of tris(hydroxymethyl)aminomethane hydrochloride, ethylenediaminetetraacetic acid, and sodium chloride (TNE) buffer to remove any remaining vaginal cells. To lyse the pellet, the researchers incubated the samples overnight in the second lysis buffer solution containing TNE buffer with 1% sodium dodecyl sulfate, 100µg/mL proteinase K, and 0.04M dithiothreitol. Finally, both vaginal DNA and sperm DNA were purified using the phenol/chloroform extraction method. DNA was precipitated using 3M sodium acetate and absolute ethanol. The authors reported that sperm cells and vaginal cells had been isolated from a mixed sample. Additionally, they found there was a possibility that sperm cells had been lysed during the first digest leading to some carryover into the vaginal cell fraction. Regardless, the authors were able to modify the original differential extraction to separate male DNA from female DNA (Yoshida et al., 1995). This modification did not address the previously discussed disadvantages, namely, the use of toxic solvents during purification and the extended incubation period. Complete separation could not be obtained for sperm and vaginal cells because of cross-contamination. Additionally, the authors did not estimate the amount of male DNA loss.

Nylon Mesh Filtration

In 1998, Chen et al. developed a physical method for isolating sperm cells from vaginal cells. Up until this point, the methods of separation discussed were chemical separation, using lysis buffer systems to lyse vaginal cells to isolate sperm cells preferentially. However, chemical separations have their limitations. As discussed in Yoshida et al. (1995), some of the sperm cells were lysed during the first lysis digest despite the lack of a reducing agent, leading to sperm cell

contamination in the vaginal fraction. Because sexual assault samples have low sperm counts, losing additional cells due to the lysis process could inhibit the identification of the perpetrator of the crime. To combat this issue, Chen et al. (1998) created a physical separation method to filter out spermatozoa from vaginal epithelial cells based on differences in size and shape. Sperm cells have a smaller, oval shape, which allows them to pass through nylon mesh filters, while the larger, flatter epithelial cells are trapped on the filter. Through this project, the authors found that 70% of sperm cells will pass through a filter with a pore size of 10μm, while only about 2% of vaginal cells will pass through. Additionally, the researchers noted but did not provide a number, that some lysed epithelial nuclei can pass through the filter into the sperm cell fraction. Despite this carryover, Chen et al. (1998) argued that this filtration process was a reliable and efficient way to differentiate spermatozoa from vaginal cells. This methodology eliminated toxic chemicals like phenol and chloroform by physically separating sperm cells from epithelial cells. However, because epithelial cells readily lyse, the filtration process allowed female DNA to pass through into the sperm fraction.

Flow Cytometry Using Antibody-Antigens

Another form of physical separation was developed by Schoell et al. (1999) to minimize the chance of obscured PCR results due to overamplified female short tandem repeats. Schoell et al. (1999) utilized flow cytometry and fluorescent multiplex PCR to isolate sperm cells from vaginal cells based on cell size and shape, surface phenotype, cytoplasm, and ploidy. Gametes such as sperm cells do not express human leukocyte antigen/major histocompatibility class I antigens until further cellular development. Leukocytes, or white blood cells, contribute to a significant percentage of vaginal cells. In addition, white blood cells have an antigen on their surface called CD45, a receptor-linked protein tyrosine phosphatase. Sperm cells, however, do not

possess this antigen. Finally, epithelial vaginal cells contain cytokeratin, while sperm cells do not. Schoell et al. (1999) exploited the differences in human leukocyte antigen/major histocompatibility class I antigens, CD45, and cytokeratin between vaginal cells and sperm cells as a means of separating them in mixed samples using fluorescently labeled antibodies. Because sperm cells do not have the necessary antigens, they did not stain while the vaginal cells were stained green.

First, the researchers washed the samples with a phosphate-balanced salt solution and fixed the cells using paraformaldehyde and saponin. Next, normal sheep serum was added to the cell suspensions to limit nonspecific reactivity before the addition of three mouse antihuman monoclonal antibodies, pan cytokeratin, clone MNF 116, IgG2a, CD45-FITC, clone 2D1, IgG1, or mouse isotype control antihuman monoclonal antibodies. Afterward, the cells were collected by centrifugation and resuspended in a solution of Ribonuclease A and propidium iodide. The suspension was incubated and filtered through a 40μm mesh. The researchers performed fluorescence-activated cell sorting on a FACSort using a 488nm argon laser and ran the cells through the piezoelectric sorter in the flow cytometer. The samples were then sorted and underwent a phenol/chloroform extraction protocol with dithiothreitol and proteinase K. Following further genetic analysis, the researchers determined that the physical separation of sperm cells from vaginal cells using human leukocyte antigen/major histocompatibility class I antigens, CD45, and cytokeratin was 36% more sensitive than the preferential lysis method. Therefore, the researchers stated that using this methodology would enhance the quantitative PCR results compared to the preferential lysis method, making it the better choice for separating sperm cells from vaginal cells (Schoell et al., 1999). While this antibody-antigen method separated the cell types more efficiently than differential extraction, Schoell et al. (1999) noted that criminalists would have to change from

vaginal swabs to vaginal lavages to collect sexual assault samples to maximize the efficiency. The use of lavages is more invasive than using vaginal swabs because lavages involve "washing" out the vaginal cavity with water or a medicinal solution.

Early Advancements Using Microfluidics and Automation

BioMek® 2000 Pilot Study

Stepping away from physical filtering and chemical modifications, Greenspoon and Ban from the Virginia Division of Forensic Science performed a pilot study using a robotic system to extract DNA from mock sexual assault samples as well as from other cell and tissue types. The robotic system, the BioMek® 2000, was used in tandem with the DNA IQ™ System in this study. In the DNA IQ™ System, silica-coated magnetic beads separate DNA from cellular debris after exposure to a lysis buffer that breaks open the cells. After attaching to the beads, the DNA is washed several times in a wash buffer before being removed from the beads using heat. After manually separating the sperm fraction from the vaginal fraction, the authors loaded the vaginal lysates and sperm pellets onto the BioMek® for DNA extraction. The DNA was removed from the magnetic beads and analyzed using the PowerPlex® 1.1 System. Based on the results from this portion of the study, the authors believed that the dual systems (BioMek® 2000 and DNA IQ™ Systems) could be used to extract DNA successfully from vaginal cell lysate and sperm pellets. Next, the authors wanted to compare the automated version of differential extraction to the manual version. Not only did the robotic system outperform manual extraction, but it also reduced the time necessary to complete the extractions. An analyst can take over 5 hours to extract the DNA from one sample using manual methods. Using the BioMek® System, 40 samples can be extracted in 1.25 hours (Greenspoon & Ban, 2002). To further illustrate the impact of robotic extraction methods, one analyst in a typical 8-hour workday could extract 256 samples using the BioMek®

System rather than the 1.6 samples they would have otherwise extracted manually. However, one significant problem is that analysts must perform the differential separation before using the BioMek® to purify the samples.

Laser Capture Microdissection

In addition to sterile cotton swabs, microscope slides have been used to determine if spermatozoa are present and to estimate the number of cells by hematoxylin and eosin staining. Following microscopic examination, if sperm cells are present, genetic analysis is carried out on the evidentiary swabs. If a DNA profile cannot be obtained from the swabs, the only remaining sample is the microscope slide from the microscopic analysis. Therefore, those slides are submitted for DNA analysis, where the sperm cells are removed using the preferential lysis method. Unfortunately, traditional preferential lysis does not work as well when extracting from slides due to the presence of female cells. Elliott et al. (2003) used laser capture microdissection to isolate cells from samples on microscope slides to evaluate its efficiency against the traditional preferential lysis method. First, the authors prepared the slides 6 to 30 months before laser capture microdissection. They determined the number of sperm cells on each slide by visually counting over the entire surface based on sperm head size, shape, and staining. The slides containing the fewest sperm cells were processed using the preferential lysis method, and then the same number of cells were extracted using laser capture microdissection. Following cell extraction, DNA was extracted from the sample cap using Qiagen ATL extraction buffer, proteinase K, and dithiothreitol. The reaction tubes were vortexed after being incubated for 2 hours upside down. Next, Qiagen AL extraction buffer and dithiothreitol were added to the tubes, which were incubated for 10 minutes. After adding ethanol, the sample mixtures were centrifuged through a Qiagen® spin column. The columns were then washed with Qiagen AW2 wash buffer and

centrifuged. Finally, the DNA was eluted off the columns using ABD TE elution buffer for three washes. Following PCR amplification and genetic analysis, the authors calculated the likelihood ratios. After comparing the likelihood ratios between the laser capture microdissection and preferential lysis methods, the authors noted that microdissection yielded a higher ratio for 15 of the 16 sample pairs. Based on these results, the authors ardently argued for the preferential lysis method to be replaced by laser capture microdissection (Elliott et al., 2003). One issue with this method was that it was highly time-consuming and labor-intensive.

Differex™ System

In 2004, Promega Corporation developed a new method of separating sperm cells from vaginal cells. The Differex™ System utilizes a proteinase K digestion of epithelial cells, differential centrifugation, and phase separation. After being digested in proteinase K, the sample and buffer solution were placed in a spin basket in a microfuge tube containing Promega's proprietary Separation Solution. While being centrifuged, sperm cells were pelleted at the bottom of the tube and remained separated from the epithelial cells because the Solution was denser than water but less dense than sperm, which acts as a protective layer. The uppermost layer, which contains epithelial DNA, was removed. Next, the Separation Solution was washed to ensure that nothing of the aqueous layer remained. According to Tereba et al. (2004), water was added to the Solution to dilute it and removed after 30 seconds without centrifugation. They also explained that approximately half of the Separation Solution can be removed, eliminating any cell debris. Once the washing step was completed, two volumes of DNA IQ™ Lysis Buffer containing dithiothreitol were added to the tube, which will lyse the sperm while solubilizing the Solution. Finally, DNA IQ™ Resin was added, which purifies the sperm fraction. The epithelial fraction was purified using the same reagents as above. While this method drastically reduced the overall time necessary to

process a sample, the same limitations as those of the original differential extraction method still exist here. The researchers found sperm carryover into the epithelial fraction and epithelial carryover into the sperm fraction. However, this method has reduced the loss of sperm cells because there are fewer washing and centrifugation steps (Tereba et al., 2004). As with Chelex®, the DNA IQ™ Resin can only bind a certain amount of DNA, which can reduce the total yield from each fraction. Additionally, this technique relied on centrifugation to create a tightly packed sperm pellet, which can be challenging to create. On top of that, the proteinase K can lyse sperm cells prematurely, leading to contamination between the two fractions.

Microfabricated Device

The physical separation methods discussed so far include laser capture microdissection, flow cytometry, and filtration. However, Horsman et al. (2005) proposed a new method using a microfabricated device to overcome the challenges that plague both physical and chemical methodologies while remaining efficient and cost-effective. The researchers simulated sexual assault samples by adding semen to buccal swabs, which were then soaked in 0.4mL of pH 7.4 phosphate-buffered saline for 1 minute. They prepared microchips using 1.1mm borofloat glass, standard photolithography, and chemical etching. The researchers set up a CCD camera on a Leitz orthoplan microscope to observe cell separation. Additionally, they used a LIVE/DEAD Viability Kit to fluorescently visualize the cells with the microscope and a xenon arc lamp. Each microchannel was rinsed with TE buffer before use. Cell separations were performed using either gravity-driven or syringe pump-driven flow methods.

After separation, the DNA was extracted using a QIAamp® DNA Mini Kit. The DNA was then amplified using a GeneAmp® PCR System 2400 Thermocycler. The Applied Biosystems™ Amp/STR COfiler multiplex amplification kit was used, followed by separation on an Applied

Biosystems™ PRISM 310 genetic analyzer. Horsman et al. (2005) discovered that while gravity-induced flow did separate epithelial cells from sperm cells, it was inconsistent due to dynamic changes in the fluid volumes during the separation process. Therefore, they proposed using a syringe pump-based flow to keep the flow rate consistent. They found that not only was the separation more straightforward to control but there was an increase in flow rate that did not dislodge any epithelial cells. Based on these results, the researchers stated that the syringe pump-driven flow method was effective at separating sperm cells from epithelial cells in roughly 30 minutes, which makes it favorable for high-throughput automation. The researchers also claimed that this method would prevent epithelial cell contamination in the sperm fraction (Horsman et al., 2005). While this study showed promising results when replacing differential extractions with a faster, cheaper, efficient, and reliable method, the most significant problem was that it used hydrofluoric acid during the etching process. Hydrofluoric acid is hazardous. An additional issue with this method was that it requires specific equipment to watch the cell separation process, which could have a massive upfront cost for forensic labs.

BioRobot® EZ1 and BioRobot® M48 Comparison

Kishore et al. (2006) attempted to optimize the DNA extraction of low-yield and degraded samples using the BioRobot® EZ1 and BioRobot® M48 systems. Both instruments utilize silica-based extraction methods and are used with single-use extraction tubes provided by the manufacturer within kits containing the necessary reagents and solutions. Kishore et al. (2006) prepared liquid blood and semen samples by diluting them in sterile phosphate-buffered saline or water. After the lysis stage, a carrier molecule, polyadenylic acid RNA or glycogen, was added to some of the samples. EZ1 DNA Tissue kit and MagAttract DNA Mini M48 kit were used before the automated extractions. Buffer G2 and proteinase K were added to the blood samples, while

Buffer G2, proteinase K, and dithiothreitol were added to the semen samples. In addition to the automated extraction methods, the researchers performed organic DNA extractions using tris(hydroxymethyl)aminomethane, ethylenediaminetetraacetic acid, sodium chloride, sodium dodecyl sulfate, and proteinase K. Dithiothreitol and sodium acetate were also used in addition to the buffers mentioned above to extract sperm cells. Following this, the lysates were extracted three times using phenol/chloroform/isoamyl alcohol. DNA quantification was performed using singleplex and multiplex assays on an ABI 7000 Sequence Detection System. Short tandem repeat typing was performed using AmpFlSTR® Identifiler™ PCR amplification. The samples were genetically analyzed using an ABI 3100 genetic analyzer. The results of the extraction efficiency study indicated that the traditional organic extraction method outperformed both robotic systems especially at low DNA concentrations. The authors noted that the poor recoveries could be due to the DNA binding capacity of the silica beads and the loss of DNA through non-specific binding to sites on the beads or even the tube walls. The researchers also found that using a carrier molecule like polyadenylic acid or glycogen increased the DNA yield by 40-fold in some instances. Additionally, they postulated that the carrier RNA blocked the sites on the tube walls, centrifugation filter, and silica bead surfaces. Furthermore, it was noted that if DNA adsorption to silica is driven thermodynamically by entropy, the RNA carrier enhances DNA adsorption by acting as a competitor for the remaining solvent water, facilitating DNA binding to the silica beads (Kishore et al., 2006). While it is unfortunate that the robotic systems did not extract DNA from sperm cells more efficiently than traditional methods, a positive outcome from this project was the discovery that adding a carrier molecule has the potential to aid in retaining as much sperm cell DNA as possible.

Optimization of Buffers

Norris et al. (2007) developed a methodology for more efficient cell elution from cotton swabs to increase sperm recovery rates. First, vaginal and buccal epithelial swabs were collected. The swabs were cut into consistently sized pieces, and 0.4μL semen was added to each sample. Norris et al. (2007) tested numerous detergents, including 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, sodium dodecyl sulfate, sodium lauroyl sarcosinate, cetyltrimethylammonium bromide, or polyethylene glycol tert-octylphenyl ether. Swabs were incubated for 2 hours at 42°C in either 10mM citrate buffer, *T. viride*-based cellulase solution, or detergent solution. Additional swabs were extracted using the conventional differential extraction method, where samples were incubated at 42°C in 1mM tris(hydroxymethyl)aminomethane hydrochloride, 1mM ethylenediaminetetraacetic acid, 10mM sodium chloride, 2% sodium dodecyl sulfate, and 20μg/mL proteinase K. After incubation, holes were poked into the bottom of the tubes. The tubes were then placed inside another tube and centrifuged for four minutes. After centrifugation, the collected lysate was vortexed and aliquoted for microscopic cell counting. Norris et al. (2007) discovered that the best detergent for eluting cells from swabs was sodium dodecyl sulfate, which had a percentage recovery of 75.6%. Additionally, sodium dodecyl sulfate had approximately two times the percentage recovery of conventional differential extraction. Further testing revealed that proteinase K decreases the percentage recovery, especially with longer incubation times. Norris et al. (2007) determined that increasing the incubation temperature over 42°C to the standard 56°C reduced the percentage recovery by 77%. Based on the above results, the researchers were confident that this elution method could be applied to forensic casework (Norris et al., 2007). Conventional differential

extraction methods use 1-2% sodium dodecyl sulfate and other buffers. The findings of the above paper show that the additional buffers may produce some degradative effects.

Comparison of Differex™ and Chelex®-100

Valgreen and Edenberger (2008) compared the Differex™ System to the commonly used Chelex®-100 method. First, the researchers prepared simulated samples by adding sperm and female epithelial cells to cotton swabs. Part of the samples was extracted using the Chelex®-100 method, while another part was extracted using the Differex™ System in four phases. Phase 1 followed the System instructions with 270µg/mL proteinase K. Phase 2 included two modifications: 1) the substrate was removed after vigorous mixing, and 2) the proteinase K concentration was increased to 500µg/mL. Phase 3 was executed the same as Phase 2; however, the incubation time was increased from 5 to 30 minutes, and dithiothreitol concentrations were increased from 6.7 to 40mM. Phase 4 was performed the same way as Phase 3; however, the separation solution was not removed. The extracts were quantified using a 7300 Real-Time PCR System and Quantifiler™ Human DNA Quantification Kit. After quantification, the extracts were amplified using the GeneAmp® PCR System 9700 with AmpFlSTR® SGM Plus® PCR Amplification kit. Finally, the amplified samples were separated using an Applied Biosystems™ 3130xl genetic analyzer. Valgreen and Edenberger (2008) explained that Phase 1 led to mixed STR profiles, suggesting incomplete separation. Phase 2 yielded profiles with the male DNA being the major contributor and the female DNA being the minor, similar to the results of the Chelex®-100 method. Microscopic examinations were performed to visualize the sperm pellet. However, the separation solution used in the Differex™ prevented even distribution of cells onto microscope slides and had to be heated before thoroughly drying. Additionally, the researchers noted that the sperm heads tended to cluster together with epithelial debris. Despite yielding comparable results

to the conventional Chelex®-100 method, the authors stated that the Differex™ System would not be implemented in their crime laboratory because of the microscopy-related issues (Valgreen & Edenberger, 2008).

RNA Carrier with Silica Monoliths

Shaw et al. (2009) developed a microfluidic-based silica monolith methodology to help enhance DNA extraction. They created silica-based monoliths by combining a solution of aqueous potassium silicate with formamide, which was injected into a glass capillary. After injection, the solution was cured overnight at 90°C to solidify the monolith. Prior to extraction, tris(hydroxymethyl)aminomethane hydrochloride/phosphate-buffered saline buffer was flowed over the monolith to activate the silica. QIAamp® DNA Micro kit was used to extract DNA of a known concentration from human buccal swabs. Next, a portion of that DNA was added to a 5M guanidine hydrochloride solution with various amounts of polyadenylic acid carrier RNA. Following this, the solution was added to the monolith at a rate of 2.5μL/minute. Next, an 80% isopropanol wash was added at 5μL/minute to remove contaminants. The added DNA was eluted with ultrapure water at 1μL/minute. DNA was quantified using Quant-iT™ PicoGreen® assay and a FLUOstar OPTIMA microplate reader. Finally, the samples were amplified using THO1 microsatellite forward and reverse primers and a Techne TC-312 thermal cycler. The researchers determined that having a ratio of 10:1 for RNA carrier-to-DNA recovered all 25ng of DNA, whereas only 5ng of DNA was recovered when no carrier was added. Based on the results, the authors noted that using a carrier RNA is suitable for microfluidic devices because the RNA preferentially binds to the silica instead of the nucleic acids (Shaw et al., 2009). This research has since been continued, which will be discussed later in the review.

Automation, Adaptions, and Applications

Holographic Optical Trapping

Chakrabarty (2010) introduced an optical trapping method that aids sperm cell separation without impeding downstream STR analysis. Holographic optical trapping is a physical separation-based technique where a hologram is created to change the spatial wavefront of a laser beam to refocus the light and form optical traps. Specifically, the wavefront is modified to split the beam into several smaller beams with fixed relative positions dictated by the hologram. The researcher noted that 5-10μL of the sample mixture, eluted from simulated forensic swabs, were used in trapping and sperm isolation. Additionally, they relied on microscopic visualization to determine if the cell trapping and subsequent cell recovery were successful. After trapping, the samples were pipetted from the recovery site and sent for DNA extraction and STR analysis. It took 3 to 4 hours for 400 sperm cells to be captured. Chakrabarty (2010) stated that the trapping process was tedious and required extensive manual labor. However, they did note that if the cells were fluorescently labeled, the process could be automated to take approximately 1 hour. After testing numerous fluorescent dyes, including Christmas tree stain, Propidium Iodide, Sybr 14, Hyliter, DAPI, and Alexa 546-NHS ester; Propidium Iodide and DAPI were the best choices based on labeling efficiency, photostability, and ease of use. Chakrabarty (2010) stated that they could recover more than 95% of sperm cells after trapping the cells with passive microfluidic cartridges. While the holographic trapping method led to a large portion of sperm cells being recovered, the most significant disadvantage was the 3-4 hours needed to capture 400 cells. The average workday is 8 hours. Therefore, the methodology should be automated before implementation into casework.

Swedish Collaborative Study

The enormous losses of male DNA have been one of the many reasons why several research projects are devoted to developing a more efficient, effective method for isolating sperm cell DNA. Vuichard et al. (2011) detailed a collaborative study involving nine Swedish laboratories—each lab using differential extraction methods to extract DNA from challenging samples. The simulated samples were created by adding 1:50 diluted semen to female buccal swabs. Four additional samples were prepared by adding fresh semen and two-year-old semen. Following this, the samples were randomized and sent to the participating laboratories. Each lab performed the first cell lysis with proteinase K and either Differex™, lysis buffer, Chelex®, or ATL buffer. The number of washing steps used by the labs ranged from 1 to 4 washes. For the second cell lysis, every lab added dithiothreitol to the lysis solutions used in the first cell lysis. The extracted DNA was purified using organic extractions, QIAamp® DNA mini kit, Chelex®, or QIAamp® DNA micro kit. Finally, the labs used Microcon filters, Centricon filters, or precipitation to concentrate the extracted DNA. After receiving the concentrated DNA extracts, Vuichard et al. (2011) quantified the extracts twice with the Quantifiler™ Human DNA kit and Human Male Y DNA Quantification kit using an ABI 7300 Real-Time PCR system. Finally, the extracts were amplified using the Applied Biosystems™ SGM Plus® Kit on a GeneAmp® 9700 thermal cycler. The amplified fragments were separated using an ABI 3100 genetic analyzer. Between all nine labs, the amount of male DNA recovered ranged from 0.0 to 26.5ng, while the amount of female DNA recovered ranged from 0.1 to 6729.6ng. Additionally, it was discovered that the differential DNA extraction obtained ~6% of the total amount recovered through direct extractions. Male DNA concentrations ranged from 0.09ng/μL to 1.06ng/μL. Finally, the authors noted that 50%-64% of female DNA and 94%-98% of male DNA were lost during the differential extraction methods used

by the laboratories (Vuichard et al., 2011). A differential extraction method must recover every sperm cell because sexual assault kit evidence has minimal amounts of spermatozoa. Therefore, a methodology that consistently contributed to the loss of 94%-98% of male DNA should not be used in a forensic setting.

Mitochondrial DNA-Based Typing

Pereira et al. (2012) developed a mitochondrial DNA-based STR typing technique for single sperm cells. First, the researchers obtained post-coital vaginal swabs and buccal swabs from five couples. DNA from the buccal swabs was extracted using the QIAamp® Mini kit. The extracted DNA was amplified using primers L15896 and H719, the forward and reverse primers for a specific genetic region within mitochondrial DNA called the D-loop region. Amplification was performed on a Biozym PTC-225 Tetrad thermal cycler. After amplification, the DNA fragments were separated using gel electrophoresis in a 1% agarose gel and TBE buffer. The PCR products were purified using the QIAquick® spin PCR purification kit. Next, the BigDye® Terminator v1.1 Cycle Sequencing kit was used to sequence the mtDNA strands. Finally, the sequenced DNA fragments were analyzed using a 3730 DNA analyzer. The post-coital vaginal swabs were soaked in sterilized water for 1 hour before the solution was allocated to microscope slides and air-dried. A DMIRB/E inverted microscope was used to observe the sperm cells. Once the preferred sperm cells were visualized, a drop of sterilized water was added to the location of the cells, which were captured using a mechanical micromanipulator and a CellTram™ Oil device. After capture, individual sperm cells were transferred to a tube containing ALT buffer from the QIAamp® DNA Investigator kit, which was then used to isolate the DNA from the captured cells. Finally, Pereira et al. (2012) amplified the extracted DNA twice using previously designed sequence-specific primers. After amplification, the samples were purified and sequenced

following the protocols outlined above for the buccal swabs. The researchers found that double PCR successfully amplified 75% of the isolated, single sperm cells. Complete male profiles were obtained for every captured cell (Pereira et al., 2012). Based on the above results, the authors argued that their mitochondrial DNA-based method was better in some instances than traditional differential extraction methods because there can be limited nuclear DNA from sperm cells in forensic samples. However, this method focused on PCR amplification while using micromanipulation to capture sperm cells—whereas other methods focused on DNA extraction. Additionally, this method relies on in-house designed primers, which can be expensive and time-consuming to design. Micromanipulation can take numerous hours because it relies on microscopic visualization of sperm cells.

Two-Step Automated Sperm Cell Recovery

Hulme et al. (2013) developed a two-step sperm cell recovery method for sexual assault evidence samples. First, two amounts, high and low, of semen were added to pre-collected buccal swabs to simulate sexual assault evidence. In addition to swabs, different fabrics encountered in sexual assault cases were tested, including cotton underwear, nylon underwear, denim jeans, nylon tights, a pullover, bedding, bath towels, and underwear liners. Eluted female buccal cells and spermatozoa were added to the fabric samples. This study used two elution methods: Cellmark's sperm elution method and Cellmark's water elution method. For the sperm elution method, the samples were incubated in Mo Lite buffer at room temperature for 30 minutes after being placed into microfuge tubes. Following incubation, the substrates were transferred to spin baskets and centrifuged for 5 minutes. Next, the supernatant was removed. Samples were placed in sterile tubes containing Mo Classic buffer and diluted proteinase K and incubated at room temperature for 2 hours. After this, the samples were sonicated, vortexed, and centrifuged for 5 minutes after being

placed in a spin basket. After centrifugation, the supernatant was removed without disturbing the pellet. 0.01M tris(hydroxymethyl)aminomethane buffer was added to the pellet. After vortexing and centrifuging, the supernatant was removed, leaving the sperm pellet. Moving onto the water elution method, samples were placed in a tube with a spin basket, and molecular-grade water was added. The samples were vortexed for 5 minutes and centrifuged for 5 minutes. The supernatant was removed. After cells were eluted from the samples, DNA was extracted using a fast differential or standard preferential extraction method. Using the fast method, the researchers added a lysis buffer made from Mo Classic buffer and proteinase K to the epithelial pellet and seminal pellet fractions, which were incubated at 56°C for 20 minutes. After incubation, the samples were centrifuged and transferred to Qiagen EZ1 BioRobot® to be further purified using the DNA Investigator kit. The SP pellets were incubated in the lysis buffer for 30 minutes and centrifuged for 3 minutes. After removing the supernatant, the sperm pellet was washed three times using sterile water. After each washing step, the samples were centrifuged, and the supernatant was removed. Following the final supernatant removal, the sperm pellets were incubated in a mixture of Mo Classic buffer, proteinase K, and dithiothreitol for 15 minutes at 56°C. After the incubation and brief centrifugation, the samples were transferred to the BioRobot® and purified using the DNA Investigator kit. The pellets eluted using the water-based method were recombined with their specific supernatant. After a 3-minute centrifugation, the supernatant was removed. The pellets were incubated in G2 buffer and proteinase K for 30 minutes at 56°C. After incubation, the samples were centrifuged, and the supernatant was transferred to a new tube and purified using BioRobot®. The sperm pellets were incubated in 1.75% sodium dodecyl sulfate and proteinase K for 30 minutes at 56°C. After centrifuging for 3 minutes, the supernatant was removed, and the pellets were washed twice using G2 buffer while centrifuging between washes. After washing, the samples

were incubated for 2 hours in G2 buffer, dithiothreitol, and proteinase K at 56°C. After centrifuging, the pellets were purified using BioRobot® and the DNA Investigator kit. Once the extraction processes were completed, the DNA was quantified using a LightCycler 2.0 and an Amelogenin assay. STR amplification was performed using the AmpFlSTR® SGM+ kit. Following amplification, the DNA fragments were separated on a 3130xl genetic analyzer. Hulme et al. (2013) discovered that the samples eluted using Cellmark's water elution method led to mixed cell pellets with both nucleated epithelial cells and sperm cells upon visualization via microscopy. However, the sperm elution method separated the cell types so that there were no nucleated epithelial cells on microscope slides prepared using the seminal pellet fraction. For the different fabric samples, the sperm elution method recovered double the number of spermatozoa that the water-based elution method recovered. STR analysis revealed that the standard preferential method yielded complete male profiles for 5 of the 6 tested, while the sperm elution method yielded complete profiles for all 6. Based on the above results, Hulme et al. (2013) argued that the two-buffer sperm elution method outperformed the conventional differential extraction method. However, their method involves numerous incubation steps that total nearly 7 hours from start to finish. The standard differential extraction method only has an incubation period of 1 hour. Therefore, it would be disadvantageous to implement a method that takes one full day to extract DNA from forensic samples.

MOSPD3-Bead Separation

Li et al. (2014) developed a bead-based separation method to isolate sperm cells from epithelial cells. First, they made cell suspensions by adding 1mL of either 10^3, 10^4, or 10^5 cells/mL sperm suspension to 1mL of 10^4 cells/mL epithelial cell suspension. Additionally, 52 vaginal swabs were obtained from rape cases, which had been preserved for 1 day, 3 days, or 10 days.

Next, the samples were incubated for 1 hour at 37°C after 0.5µL biotin-labeled MOSPD3 antibody was added. After incubation, the samples were centrifuged for 8 minutes. The supernatant was removed, and the sperm pellet was washed with phosphate-buffered saline three times. Dynabeads FlowComp Flexi was added to the sperm pellets and incubated for 15 minutes at 5°C. After incubation, the samples were placed on a magnetic stand for 5 minutes. After being washed three times with phosphate-buffered saline, the sperm cells were removed from the beads using a release buffer. The dried vaginal swabs were incubated in phosphate-buffered saline for 1 hour at 37°C. After placing the swabs on a centrifugation filter, the researchers centrifuged the samples for 2 minutes. Once the pellets had been washed three times with phosphate-buffered saline, sperm cells were isolated using the same MOSPD3-bead protocol described above. DNA was extracted from the sperm cells using a QIAquick PCR purification kit. Quantification was performed using a spectrophotometer. Amplification was performed using AmpFlSTR® Identifiler® Plus PCR amplification kit. Finally, the amplified fragments were separated using a Hitachi High-Technologies 3130XL genetic analyzer. Li et al. (2014) obtained complete male-only STR profiles for the 30 samples with varying sperm cell concentrations. Sperm cells were successfully detected for the dried vaginal swabs at rates of 100%, 87.5%, and 40% for 1 day, 3 days, and 10 days, respectively. Despite the success of the immunomagnetic beads, the authors mentioned that this method needed to be optimized, and more stable sperm cell surface antigens needed to be found. However, they argued that this method could replace conventional differential extraction protocols (Li et al., 2014). Traditional bead-based methods, such as Chelex®, have a set binding capacity, which can prevent 100% recovery of sperm cells since cellular debris or epithelial DNA can bind to them. Using sperm-specific antibodies does reduce this effect because only cells with the required antigens can be captured. However, there is still a chance for sperm cell loss during the

numerous washing steps since the antigen-antibody interactions may not be strong enough to hold the sperm cells.

Antibody-Magnetic Bead-Based Separation

Grosjean and Castella (2015) developed an antibody-magnetic bead-based method using CD52, a glycoprotein found on the heads of sperm cells. First, the researchers created simulated sexual assault samples by adding previously diluted sperm cells to buccal swabs. After being stored for 6 months, the swabs were incubated in 800μL phosphate-buffered saline and 2mM ethylenediaminetetraacetic acid for 30 minutes at 25°C. After incubation, the samples were centrifuged. The pellets were stained for 15 minutes at 4°C in 50μL phosphate-buffered saline/ethylenediaminetetraacetic acid and 8μL CD52-magnetic beads. After being washed and resuspended in 150μL and 300μL phosphate-buffered saline/ethylenediaminetetraacetic acid, respectively, the stained pellets were transferred to columns, which had been rinsed with 500μL phosphate-buffered saline/ethylenediaminetetraacetic acid twice. Spermatozoa were retained on the column, while non-sperm cells flowed through and were collected. The columns were washed twice with 500μL phosphate-buffered saline/ethylenediaminetetraacetic acid and removed from the magnetic field. Next, the captured sperm cells were eluted from the columns using 700μL phosphate-buffered saline/ethylenediaminetetraacetic acid. After collection, the samples were centrifuged and retained in 200μL phosphate-buffered saline/ethylenediaminetetraacetic acid. Sperm cell DNA was extracted using the QIAamp® kit. Amplifications were performed using AmpFlSTR® NGM® SElect kit. Finally, amplified fragments were separated using an Applied Biosystems™ 3130xl genetic analyzer. Grosjean and Castella (2015) stated that after the captured fractions were stained with propidium iodide and analyzed with flow cytometry, the non-sperm fractions had diploid peaks, while the sperm fractions had haploid peaks. Additionally, the authors

noted that the CD52-magnetic bead-based method yielded usable STR profiles. They also explained that a preliminary study was performed involving post-coital samples. While they did not give the study's results, they still claimed that this methodology has real-world applicability (Grosjean & Castella, 2015). While the above results are promising, further projects need to be explored using this protocol with simulated sexual assault samples compared to conventional methods before any conclusions can be made. The problems plaguing Li et al. (2014) discussed in the previous section also apply here.

Fluorescence- and Magnetic-Activated Cell Sorting

Xu et al. (2016) developed a protocol involving fluorescence- and magnetic-activated cell sorting to isolate sperm cells from simulated sexual assault samples. Cell suspensions were prepared with 10^7 cells/mL densities. Mock samples were created by mixing 5μL semen from two donors and adding the mixture to pre-collected vaginal swabs. After this, the samples were placed in phosphate-buffered saline to create cell suspensions. Additional samples were created for a sensitivity study where diluted sperm cells were mixed with vaginal cells to create a dilution series, including 1:1, 1:4, 1:16, 1:32, and 1:64. Another dilution series was created for the sensitivity study using diluted B and O blood type sperm cells. This series had 1:1, 1:2, 1:4, 1:8, and 1:16 dilutions. Fluorescein isothiocyanate-conjugated A kinase anchor protein 3 (FTIC-conjugated AKAP3) polyclonal antibody was mixed with Anti-FITC MicroBeads. After incubating for 2 hours at 20°C, the beads were washed thrice with phosphate-buffered saline. Next, the AKAP3-magnetic beads were mixed with the cell suspensions. The samples were incubated in phosphate-buffered saline containing 10% fetal bovine serum and 2mM ethylenediaminetetraacetic acid for two hours at 37°C. Following incubation, the samples were washed thrice with 1.5 mL phosphate-buffered saline and centrifuged for ten minutes. MS Columns and MACS Separator were used to complete

the separation process. Flow cytometry was used to detect the FTIC-labeled cells. For fluorescence-activated cell sorting, sperm mixtures were filtered through a 40μM Cell Strainer. FITC-labeled blood group A or FITC-labeled blood group B antibodies were added to the sperm cell suspensions and incubated for one hour at 37°C in phosphate-buffered saline containing 10% fetal bovine serum and 2mM ethylenediaminetetraacetic acid. After incubation, the samples were washed with phosphate-buffered saline and centrifuged for 10 minutes. Finally, a FACSAria™ II sorter was used to sort the labeled cells. A DNA IQ™ kit was used to extract DNA from the magnetic-activated cell sorting and fluorescence-activated cell sorting samples. Quantification was performed using the Quantifiler™ system. Next, the extracts were amplified using AmpFlSTR® Identifiler® Plus PCR amplification kit with a GeneAmp® PCR System 9700. Finally, the amplified fragments were separated using an ABI Prism® 3130xl genetic analyzer. Xu et al. (2016) stated that the magnetic-activated cell sorting separation led to 86% of the total sorted cells being sperm cells, whereas the fluorescence-activated cell sorting separation led to 90%. For the sensitivity studies, magnetic cell sorting led to full STR profiles for the 1:1, 1:4, 1:16, and 1:32 sperm-to-vaginal dilutions. Fluorescence cell sorting led to full STR profiles for the 1:1, 1:2, 1:4, and 1:8 sperm of B-type-to-sperm of O-type dilutions. Finally, when the mock samples were separated using magnetic-activated cell sorting followed by fluorescence-activated cell sorting, the percentage separation of A-type sperm cells from B-type increased from 34.2% to 81.6%. One issue with this method, which the authors brought up, is using ABO antibodies with fluorescence-activated sorting. If a mixture of sperm cells has the same blood type, the cells will not be separated using fluorescence cell sorting. Additionally, both magnetic- and fluorescence-activated cell sorting require intact sperm cells for separation. If spermatozoa are prematurely lysed during

digestion, DNA will be lost in the non-sperm cells. As a result of these challenges, the authors believe this method requires further optimization before adoption by crime labs (Xu et al., 2016).

Direct-to-PCR Method

Tobe et al. (2017) validated a direct-to-PCR method that bypassed the traditional differential extraction protocol and developed a differential isolation method for sexual assault cases. To simulate sexual assault evidence, Tobe et al. (2017) allocated semen dilutions onto sterile cotton, which was allowed to air dry before direct PCR analysis. The dilution series included 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160. Additionally, the researchers included stains from previous proficiency tests from the German DNA profiling (GEDNAP) group. Neat semen added to different substrates was used in this study. Samples containing a mixture of semen and saliva were incubated in a Promega Swab solution and proteinase K at 70°C for 15 minutes, 30 minutes, and 60 minutes. Then, the samples were centrifuged, and the supernatant was removed and sent for direct PCR. The sperm fraction was washed twice with an additional Swab solution. For the samples on toilet paper and tissue, two to three fibers were collected and placed in the PCR reagents. Finally, the entire sperm fraction was added to the PCR reagents, while only 8μL of the non-sperm fraction samples were added. Amplification was performed using the PowerPlex® ESX 16 and 17 kits on a 2720 thermal cycler. The amplification products were separated and analyzed using a 3130 genetic analyzer. After PCR, Tobe et al. (2017) centrifuged and collected 10μL from the bottom of the tubes for hematoxylin and eosin staining to see if any intact sperm cells remained. After staining the post-PCR products, they found no intact sperm cells. Additionally, they reported an average global balance of 0.84 and an average local balance of 0.6 for the semen dilution samples. For the GEDNAP stains, the sample extracted using traditional methods had a global balance of 0.928, while the direct PCR method had a global balance of 0.925. However, the most

considerable difference between the two methods can be seen in the average peak heights, which were 222.65 and 1553.79 for the direct PCR and traditional methods. Moving to their proposed differential isolation method, the researchers found that the non-sperm cell components were digested fully after 60 minutes. Finally, they explained that the global balance ranged from 0.78 to 0.87 and 0.78 to 0.9 for liquid and dried mixtures on cotton substrates, respectively. Based on the above results, Tobe et al. (2017) were confident that direct PCR methods in tandem with their isolation method could produce complete male profiles even with an excess of non-male DNA. While the results of this study are promising, they fail to account for complex mixtures with more than one male donor.

DEPArray™ System

Williamson et al. (2018) tested the DEPArray™ system to prove that it would increase the recovery of sperm DNA while eliminating the need for differential extractions. The researchers created three sample sets. The first set was four cotton swabs with single-source semen approximately fifteen years old, which were stored at -20°C after being collected. The second set was made from whole blood and epithelial and sperm cells. These samples were created by diluting semen-to-epithelial cells and semen-to-whole blood-to-epithelial cells. The two dilutions ranged from 1:1 to 1:10,000 and 1:1:1 to 1:1:100. Finally, the third set was post-coital samples collected at varying times from 12 to 96 hours post-coitus. Sample and instrument preparation was performed using the DEPArray™ Forensic Sample Prep kit, DEPArray™ Manipulation buffer, DEPArray™ A300 K DS V2.0 cartridge, and SBLysePrep™ kit. The system procedures had four steps. Step 1 consisted of incubating the samples for 2 to 24 hours to release the cells. Step 2 involved centrifuging the cells to concentrate and staining them using four stain-antibody conjugates, unique for epithelial cells, sperm cells, white blood cells, and nuclei. The stains were

fluorescein, allophycocyanin, phycoerythrin, and 4',6-diamidino-2-phenylindole, respectively. Instrument preparation occurred in Step 3, where the samples were washed and added to the DEPArray™ cartridge. Finally, Step 4 consisted of targeting cells using the Cell Browser and selecting them for recovery into tubes using the Recovery Manager software. Williamson et al. (2018) performed conventional differential extractions to compare the efficiency of the DEPArray™. Samples were incubated for 1 hour at 56°C in 1000mM tris(hydroxymethyl)aminomethane hydrochloride, 500mM ethylenediaminetetraacetic acid, 5000mM sodium chloride, 20% sodium dodecyl sulfate, and 10mg/mL proteinase K. After incubation, the samples were placed in spin baskets and centrifuged. Next, the supernatant was removed, while the sperm pellets were washed three times with the digestion buffer. The final wash was performed using nuclease-free water. The sperm and non-sperm cell fractions were purified using the DNA IQ™ Casework Pro kit and Maxwell® 16. The sperm fractions were incubated at 56°C for 30 minutes in 18mg/mL proteinase K, 1-thioglycerol, and Casework Extraction Buffer. After incubation, the lysis buffer was added to both fractions, which were transferred to the Maxwell® 16. Finally, the extracted samples were concentrated using DNA Fast Flow Microcon® centrifugation filters. Quantification was performed using Plexor HY Human and Male DNA Quantification kit on a Roche Light Cycler 480II. The samples were amplified using the Life Technologies Veriti® thermal cycler using a Promega PowerPlex® Fusion 6c human DNA amplification kit. Finally, the amplification fragments were separated using a ThermoFisher Scientific 3500 x L genetic analyzer. Williamson et al. (2018) discussed that the reproducibility samples yielded the expected STR profiles. The DEPArray™ recovered sperm cells from each post-coital sample. While both DEPArray™ and the conventional differential extraction method led to mixtures in four out of five samples, the former yielded STR profiles where the major contributor

was male. For the mock samples, spermatozoa were identified in 84% when using the DEPArray™. Of the 84%, 96.2% led to single-source profiles. Complete male-only profiles were developed from all semen-to-epithelial cell dilution series, except for the 1:10,000 dilution, whereas only 12.5% of the differential extracted samples yielded single-source profiles. Finally, the DEPArray™ system yielded all single-source male-only profiles for all semen-to-blood-to-epithelial cell dilutions. Based on the above results, Williamson et al. (2018) emphatically argued in favor of this system for use in forensic science settings. While the system did reduce the carryover of female DNA to the sperm fraction, making profile interpretation easier, the upfront cost of implementing this system could be more than what a forensic crime lab is willing to spend. More importantly, if a crime laboratory used a later model of the DEPArray™ and an instrument error occurred, there is no way to remove the evidentiary sample from the cartridges.

Optical-Based Trapping

Auka et al. (2019) proposed an optical-based method for trapping sperm cells from whole sperm samples and mixed cell samples. The optical method utilizes an optical tweezer, a compact, tightly focused laser beam, which uses an immersion objective lens on an inverted microscope to form an optical trap. This optical trap forms a focal point using dielectric particles. Sample particles are moved when the laser beam is moved. Semen samples were prepared in dilution using bovine serum albumin. Vaginal cells were eluted from the swabs using double-distilled water. Mock sexual assault samples were created using equal volumes of vaginal cell and semen dilutions. The researchers built the optical trapping device using an inverted microscope fixed to a vibration isolation table where brightfield illumination was produced using an LED white light fixed approximately ten centimeters above the microscope objective. The microscope stage was motorized and could be controlled using a joystick. The researchers used an oil immersion 100X

magnification objective and a 700mW, 1064nm continuous wave laser for trapping. The stage was moved using the joystick until the laser was over the individual sperm cells. The tweezer was used to isolate and move the sperm cell to the edge of the sample droplet. Finally, this process was repeated until the required number of cells had been captured. A borosilicate glass capillary collected the isolated sperm cells via capillary action. The collected solution was pipetted onto a coverslip using a FemtoJet microinjector. After verifying that the correct number of sperm cells had been transferred, the coverslip was placed into a microcentrifuge tube until further DNA analysis. The QIAamp® DNA Investigator kit was used for the DNA extraction process. The Quantifiler™ Trio DNA Quantification kit and an ABI Prism® 7500 real-time PCR instrument were used for the quantification step. The AmpFlSTR® Identifiler Plus PCR Amplification kit and the ProFlex PCR system were used for the amplification process. Finally, the DNA fragments were separated using the 3130 genetic analyzer. The researchers determined that the optical trapping technique had sperm cell recovery from mixed samples ranging from 58% to 140%. Additionally, they stated that they obtained clean DNA profiles from six of the nine mixed samples. Therefore, based on the above results, the researchers argued that this optical tweezer-based trapping method was well suited for forensic casework. While this method is promising, the upfront associated costs with this technique could be cumbersome for smaller forensic laboratories. Also, lasers can be dangerous and require extra training to be used safely.

Nanofiber, Carrier Sperm, and Microfluidic Separation

Nanofiber Mesh

Smith (2020) performed three different protocols using electrospun nanofiber mesh (ENM) to increase the recovery of sperm cells from simulated sexual assault samples. ENMs to be used as filters were made from polycaprolactone and varied in the number of layers by being electrospun

onto molds. Each layer was laid perpendicular to the previous layer. A Hitachi TM3000 scanning electron microscope was used to determine the pore size of the meshes. Next, a magnesium oxide slurry was made to determine if sperm cells could pass through the ENMs because magnesium oxide molecules are approximately 2.8μm in diameter, which closely matches the diameter of sperm cells (3μm). In addition to the ENMs, polycaprolactone nanosieves were created to determine if physical separation of sperm cells from epithelial cells could be achieved. After the sieves had been made, a laser was used to etch 10μm holes into the polymer. Unfortunately, the polymer melted during the cutting process, leaving holes that were not uniform in shape or size. A third study was performed to determine if ENMs could be used inside microfuge tubes to capture spermatozoa. First, neat semen was added to a 6-layered ENM and imaged using the Click-iT™ EdU Cell Proliferation kit for Imaging by following the included protocol. To fix the sperm cells, 3% bovine serum albumin in phosphate-buffered saline was used to wash the samples twice. Next, 0.5% Triton X-100 in phosphate-buffered saline was added to the mesh. After incubating for 10 minutes at room temperature, the sample was incubated for 30 minutes at room temperature in Click-iT™ stock solution. Once the second incubation was completed, the stock solution was removed, and 3% bovine serum albumin/phosphate-buffered saline was added to wash the sample. After incubating at room temperature for 5 minutes, the sample was rewashed using phosphate-buffered saline. Next, Hoechst solution was added, followed by a 15-minute incubation period. Finally, the cells were visualized using a fluorescence microscope. To expedite sample visualization, Smith (2020) performed a minor test to eliminate the numerous washing and incubation steps. They determined that the sperm cells fluoresced regardless of the washing/incubation steps; therefore, these were not performed for the rest of the project. It was determined that there were too many spermatozoa in the neat samples to count physically, so a 1:3

semen dilution was prepared and fluorescently dyed. After the attempt to count spermatozoa from the 1:3 dilution failed, Smith (2020) created a 1:6 dilution and was able to count the amount of sperm cells after being fluorescently dyed. Next, they washed the samples by pipetting deionized water up and down. Following this, the cells were re-visualized using a microscope and counted to see how many cells remained. Simulated sexual assault swabs were created using a 1:50 semen dilution pipetted onto pre-collected buccal swabs. After drying for 2 hours, the samples were stored at room temperature until further analysis. Next, a 6-layer ENM was added to each sample microfuge tube. After adding the swab tips, a lysis buffer was added. The samples were incubated at 50°C for 45 minutes. Following incubation, the samples were transferred to spin baskets and centrifuged for 2 minutes. The supernatant, swabs, and spin baskets were removed without disturbing the sperm pellet. Lysis buffer with dithiothreitol was added to the sperm pellets and incubated at 70°C for 30 minutes. After this, DNA IQ™ resin was added to the non-sperm and sperm fractions. Once the samples had been incubated for 5 minutes at room temperature, a magnetic stand was used to retain the resin at the bottom of the tubes. The supernatant was removed. Lysis buffer was added, vortexed, and removed from the samples. Next, DNA IQ™ wash buffer was added and vortexed to remove leftover epithelial cells. After returning to the magnetic stand, Smith (2020) removed the wash buffer and repeated the washing step twice more. After the final wash, the tube caps were left open so the samples could air dry for 5 minutes. DNA IQ™ elution buffer was added, and the samples were incubated for 5 minutes at 65°C. After being vortexed, the samples were placed on a magnetic stand. Finally, the supernatant was removed and retained for quantification, amplification, and genetic analysis. Quantification was performed using an Applied Biosystems® Quantifiler™ Human DNA Quantification kit and a CFX96 Touch Real-Time PCR Detection System. Amplification was performed using an Applied Biosystems®

GlobalFiler™ PCR Amplification kit and a GeneAmp® PCR System 9700. Finally, the amplified fragments were separated using an Applied Biosystems® 3500 genetic analyzer. Smith (2020) determined that ENM pore size inconsistently decreased with increasing layers, affecting cell capture capability. As stated previously, when the polycaprolactone film was etched using a laser, the film melted, leading to inconsistently shaped holes. Smith (2020) also noted that the smallest diameter that could be produced was 150µm, which was 15 times the desired diameter. During the sperm retention part of this project, it was determined that the nanofiber meshes retained 42% of sperm cells after a washing step was performed. For the third study, DNA was successfully extracted and quantified from the sperm fractions. However, samples yielded mixed profiles, meaning that separation was incomplete. The researcher attempted several modifications to incubation temperatures and lengths, reagent types and volumes, and supernatant removal. Despite these attempts, mixed profiles were still generated. While it was not possible to generate single-source profiles from the sperm fraction, the author noted that using ENMs increased the sperm recovery rate up to 21 times the rate of conventional differential extraction methods (Smith, 2020). This method appears promising; however, the issue of mixed profiles remains.

Acoustic Cell Separation

Sun et al. (2021) developed a two-step acoustic cell separation method for on-site sperm cell separation. A mock sample was created by mixing epithelial and sperm cells to obtain a 25:1 epithelial:sperm dilution. An aliquot of the mock samples was added to the system via the sample inlet. Flow cytometry was used to determine the number of collected cells from the two outlets. An additional sample was created by adding 500µL of the 25:1 dilution to cotton gauze to simulate semen stains. After being air dried at room temperature for 24 hours, cut pieces of gauze were incubated overnight at 4°C in normal saline after being shaken for 2 hours at 900 rotations per

minute. Next, the solution was transferred to another tube and centrifuged for 5 minutes. The supernatant was removed, and the pellet was resolubilized in normal saline. The resolubilized pellet was added to the device, and flow cytometry was used to count the number of cells after separation. Sun et al. (2021) discovered that 88% of the sperm cells from the mock sample were isolated from epithelial cells. Additionally, they found that within the sperm fraction, only 0.4% were epithelial cells. For the seminal stain, 91% of the total recovered cells were sperm cells, while 9% were epithelial cells. This corresponded to a recovery rate of 84%. Based on the above results, the authors argued that the portable device was advantageous over conventional methods (Sun et al., 2021). One issue with this system would be the upfront cost of obtaining the device and training personnel.

Carrier Sperm

In 2022, a research study was created to develop a methodology that improved sperm cell yield while providing a cost-effective, not labor-intensive, and rapid addition to conventional differential extraction. Lansdale (2022) chose two animal sperms because genetic analytical techniques are human-specific; therefore, the added sperm would not convolute the genetic information produced during DNA analysis. A standard curve was created to identify the dilutions that contained around one hundred sperm cells because one hundred sperm cells correlate to a theoretical DNA concentration of 0.3ng. 0.3ng is considered to be a low copy number and simulates the low amount of male DNA found in sexual assault casework. DNA extraction and purification were performed using the DNA IQ™ kit from Promega® Corporations by following the protocol given with the kit; however, the research made two modifications corresponding to the animal seminal fluids. First, the carrier horse samples were created by adding both 20μL of human semen and 50μL of horse semen before the addition of lysis buffer. Second, the carrier

salmon samples were created by adding 50µL of salmon sperm before the wash buffer was added during all three wash cycles. In addition to performing an extraction with the DNA IQ™ kit, the researcher conducted an organic phenol-chloroform extraction because the resin used in the kit will only bind a certain amount of DNA; therefore, by using an organic extraction method, the researcher was able to remove the uncertainty around the binding capacity. The organic extraction was implemented following standard protocols with two modifications: 50µL of horse semen or 50µL of salmon sperm DNA was added to each 20µL human semen sample, and the stain extraction buffer volume was changed from 225µL to 175µL. Then, the samples were quantified using a Thermo Scientific NanoDrop™ 2000c Spectrophotometer and an Applied Biosystems Quantifiler™ HP DNA Quantification kit. The average DNA concentration was higher when using the NanoDrop™ compared to the Quantifiler™ HP kit; however, this is to be expected since the HP kit is human-specific, while the NanoDrop™ is not human-specific. Additionally, the average DNA concentrations were relatively close between the two types of extraction methods. After quantification, the samples were amplified using the Applied Biosystems GlobalFiler™ PCR Amplification kit and subjected to capillary electrophoresis on an Applied Biosystems™ 3500 genetic analyzer. GeneMapper *ID-X* Software was used to analyze the data to generate the electropherograms for each sample (Lansdale, 2022). After interpreting the data generated, it was determined that there was an average percentage decrease of 99.45% and 98.36% of human sperm cells for the horse modification and salmon modification, respectively, when using the DNA IQ™ extraction kit. Alternatively, there was an average percentage decrease of 88.40% for the horse modification, while the salmon modification had a percentage decrease of 84.25% when using the organic phenol-chloroform extraction. Unfortunately, based on the percentages listed above, the

hypothesis that a carrier could be used to decrease the loss of human male DNA was not supported because of low DNA concentrations in the samples.

Microfluidic System

Woolf et al. (2023) developed a microfluidic system that could replace conventional differential extraction methods while automating the process. Two tests were performed to determine the removal of epithelial cells and sperm cells from the device. First, the cotton tips from pre-collected buccal swabs were removed and quartered. Next, individual cuttings were placed in the swab chambers on the disc. The cuttings were incubated in an epithelial-cell (e-cell) lysis buffer (10µL 10X orange buffer, 2µL *prepGEM*, 100µL water) at 52°C for 5 minutes. After incubation, the epithelial fraction was manually removed. The remaining pellet was washed thrice using purified water. After washing, the pellet was incubated in the e-cell lysis buffer at 75°C for 3 minutes. Quantification was performed using a NanoDrop™ 3300 Fluorospectrometer and the PicoGreen® dsDNA assay. The sperm samples were created by adding 1:150 sperm dilution to cotton swabs, which were air-dried at room temperature for 48 hours before processing. Following this, the samples were cut and sealed in the microfluidic device. Next, each cutting underwent the same process described above for the epithelial samples. However, the researchers removed a portion of each fraction to determine the loss of sperm cells during the first elution and subsequent washing steps. The remaining portions were treated with *prepGEM* and *Acrosolv* in the orange buffer. Quantification was performed using a SensiFAST™ Probe No-ROX kit and a QuantStudio™ 5 Real-Time PCR System for Human Identification. For the epithelial samples, it was determined that most of the cells were found in the non-sperm fraction and the first wash elution. However, the remaining two washing fractions and the sperm fraction showed little to no cells remaining. The same outcome was seen for the sperm samples. Woolf et al. (2023) also found that the sperm

cells remained intact until the lysis step with *Acrosolv*. Aside from the above results, the authors noted that sperm recovery was greater than or equal to 90%. The results of the above study are promising; however, further testing is required with simulated sexual assault samples before it can be implemented into casework.

Concluding Remarks

While the above literature review is detailed, it is not a complete listing of every advancement made over the past forty years since differential extraction's inception. However, there are studies discussed that form the basis for this thesis. A few aforementioned projects that utilized polyadenylic acid RNA as a carrier molecule also inspired using a carrier to improve the recovery of human sperm DNA. Using robotic extraction techniques, Kishore et al. (2006) used polyadenylic acid to purify DNA from biological stains. Compared to traditional organic extraction techniques, robotic extractions recovered low DNA concentrations; however, when polyadenylic acid was added after cell lysis, the DNA yield increased 20-fold. (Kishore et al., 2006). Further research into this type of carrier successfully extracted DNA from buccal swabs using microfluidic silica monoliths (Shaw et al., 2009). Shaw et al. (2009) found that adding polyadenylic acid to the microfluidic device increased the DNA yield 5-fold. In 2022, Lansdale applied this carrier concept to extract male DNA from semen samples. Instead of polyadenylic acid RNA, they used horse or salmon semen as the carrier, with the horse sperm cells added before the lysis buffer and the salmon sperm DNA added during the washing steps. Lansdale (2022) sought to test two hypotheses. First, the addition of carrier sperm cells through the entire extraction process will reduce the loss of human DNA, and second, the addition of carrier sperm DNA would increase the yield of human sperm DNA during each washing step during extraction. Unfortunately, both hypotheses were refuted because adding horse sperm cells or salmon sperm DNA resulted in a percentage decrease

in human DNA of 99.45% and 98.36%, respectively when extracted with the DNA IQ™. This was confirmed during a secondary study conducted with phenol/chloroform extractions, which yielded a percentage loss of 88.40% and 84.25% for horse sperm cells and salmon sperm DNA, respectively (Lansdale, 2022). However, it is important to note that neither of the above research projects extracted human DNA from sexual assault samples, nor did they perform a traditional differential extraction.

Based on the above research studies, it is proposed that the addition of horse semen prior to differentially extracting simulated sexual assault samples will increase the human male DNA yield. Because a human-specific kit will be used for quantification, horse semen will not be detected during downstream analyses.

Introduction

Sexual assaults are crimes with one of the lowest reporting rates in the United States (Thompson & Tapp, 2023). One reason for this may be caused by the backlogs of unsubmitted and untested sexual assault evidence kits in crime laboratories (Ritter, 2011; Strom et al., 2021). Over the years, different acts such as the Violence Against Women Act have increased the funding for laboratories to process the backlog. More DNA analysts have been hired to help process the kits by working overtime. Furthermore, scientific advancements such as the automation of differential extractions have been promising; however, many of these advancements have not been implemented in forensic laboratories due to difficulty of use, associated costs, and similar male DNA yields compared to conventional separation techniques. Despite numerous funding and scientific efforts, the sexual assault crime rate has not diminished an appreciable amount. The sexual assault crime rate was 1.6 in 2000 and 1.9 in 2022 (Bureau of Justice Statistics, n.d.). The Bureau of Justice Statistics (n.d.) calculates the rate of victimization by multiplying the number of

victimizations by 1,000 and dividing that product by the number of persons aged 12 years or older. While the rate of sexual assaults has remained relatively the same for 22 years, the response to sexual assault crimes can be changed. Approximately 21% of sexual assaults are reported to police every year (Thompson & Tapp, 2023). The low reporting has been perpetuated by the perception that it does not matter if the victims report their assaults. One way to change this perception is to make sexual assault kit testing easier and faster to determine who the perpetrator is and to hold them accountable.

For nearly four decades, numerous research projects have been devoted to developing differential extraction protocols to retain as many sperm cells as possible while efficiently removing epithelial cells. In the 1980s, Gill et al. developed the first differential extraction method, preferential lysis, to separate epithelial DNA from sperm cells. Two research groups in the 1990s modified Gill et al.'s (1985) method by using milder reagents during the lysing step and by performing an extra washing step to ensure that epithelial cells had been removed (Wiegand et al., 1992; Yoshida et al., 1995). Additionally, Chen et al. (1998) executed a filtration-based method to separate sperm cells from epithelial cells based on their differences in size and shape. In the 2000s, researchers explored numerous avenues to increase the sperm DNA yield, such as antibody/antigen-based capture, robotic-based purification, laser capture microdissection, Differex™ System, microfabricated devices, microchip-based separation, enzymatic digestion, Nanotraps, dielectrophoretic separation, and carrier RNA (Greenspoon & Ban, 2002; Elliott et al., 2003; Tereba et al., 2004; Horsman et al., 2005; Voorhees et al., 2006; Valgreen & Edenberger, 2008; Shaw et al., 2009). The 2010s continued the trend of automation and antibody/antigen capture as well as the introduction of optical-based trapping methods and fluorescence-based cell sorting (Chakrabarty, 2010; Vuichard et al., 2011; Pereira et al., 2012; Hulme et al., 2013; Li et

al., 2014; Grojean & Castella, 2015; Xu et al., 2016; Tobe et al., 2017; Williamson et al., 2018; Auka et al., 2019). Finally, the 2020s contained projects that automated differential extraction, utilized bead-based separation, carrier sperm methods, DNA/RNA co-extractions, Nanofiber mesh separation, and direct-to-amplification sperm lysis methods (Smith, 2020; Sun et al., 2021; Lansdale, 2022; Woolf et al., 2023). Despite the numerous decades and research projects, forensic laboratories continue to use conventional differential extractions even though there are several improved protocols that could be implemented instead. The reasoning behind this is that perfection is expected due to the sensitive nature of accurate forensics. Because results from DNA extractions are taken to a courtroom and presented to a juror, any methodology employed is required to work 100% of the time while being correct 100% of the time. To this day, perfection remains unachievable.

The aforementioned projects that utilized polyadenylic acid inspired using a carrier to improve the recovery of sperm DNA. Polyadenylic acid, a synthetic homopolymer, is made of a single-stranded sequence of adenine nucleotides (Li, 2021). Polyadenylic acid tails are created in nuclei during polyadenylation for transcription termination, mRNA stability, and translation. In addition, polyadenylic acid has been incorporated into other research, such as carrier DNA/RNA, to aid in the precipitation of DNA and RNA (Li, 2021). Using robotic extraction techniques, Kishore et al. (2006) utilized polyadenylic acid to purify DNA from biological stains. Compared to traditional organic extraction techniques, robotic extractions recovered low DNA concentrations; however, when polyadenylic acid was added after cell lysis, the DNA yield increased 20-fold (Kishore et al., 2006). Further research into this type of carrier successfully extracted DNA from buccal swabs using microfluidic silica monoliths (Shaw et al., 2009). Shaw et al. (2009) found that adding polyadenylic acid to the microfluidic device increased the DNA

yield 5-fold. While these projects are promising, forensic laboratories do not use robotic techniques or microfabricated silica monoliths in casework. In 2022, Lansdale applied this carrier concept to extract male DNA from semen samples. Instead of polyadenylic acid, they used horse or salmon semen as the carrier, with the horse sperm cells added before the lysis buffer and the salmon sperm DNA added during the washing steps. DNA IQ™ and phenol/chloroform/isoamyl alcohol extraction methods were used because they are traditionally used in forensic laboratories. Lansdale's (2022) project sought to test two hypotheses. First, the addition of carrier sperm cells through the entire extraction process will reduce the loss of human DNA, and second, the addition of carrier sperm DNA would increase the yield of human sperm DNA during each washing step during extraction. Unfortunately, both hypotheses were invalidated because adding horse sperm cells or salmon sperm DNA resulted in a percentage decrease in human DNA of 99.45% and 98.36%, respectively when extracted with the DNA IQ™. This was confirmed when a secondary study was conducted using phenol/chloroform extractions, which yielded a percentage loss of 88.40% and 84.25% for horse sperm cells and salmon sperm DNA, respectively (Lansdale, 2022). However, it is important to note that neither of the above research projects extracted human DNA from sexual assault-type samples. Therefore, we sought to repeat the carrier sperm method using simulated sexual assault samples, which were extracted using the Oklahoma State Bureau of Investigation's differential extraction protocol followed by purification via phenol/chloroform/isoamyl alcohol extractions with ethanol precipitation. Horse semen was added prior to differentially extracting the samples.

Methodologies

Epithelial Sample Collection

Human buccal, epithelial cells from a single donor were collected using sterile cotton swabs by vigorously rubbing the inside of the cheek while rotating the swab for 30 seconds. The swabs were placed in sterile microfuge tubes and air-dried at room temperature in a sterile fume hood (Vuichard et al., 2011). Once the swabs were completely dried, the sticks of the cotton swabs were broken to close the cap on the tubes. At least two hours occurred between successive samples to reduce inter-sample variation (Vuichard et al., 2011).

Semen Sample Dilution

Human seminal fluid from a non-vasectomized, single donor was purchased for this research. Dilutions were serially made in triplicate, using the following dilution range: 1:1 (or neat), 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Semen was diluted in Ultrapure distilled water. The above range was chosen because Vuichard et al. (2011) estimated that the male-to-female DNA ratio before differential extraction ranged from 1:38 to 1:339. Each dilution was vortexed before each aliquot was removed to ensure the mixture was homogenous.

Simulated Sexual Assault Swab Preparation

After thorough vortexing, 50µL of every dilution was pipetted onto two pre-collected buccal swabs each (Vuichard et al., 2011). The swabs were then dried at room temperature in a sterile fume hood for at least 2 hours before being sorted into two different groups and stored in sterile microfuge tubes in the -20°C freezer until further analysis (Alderson et al., 2018; Katilius et al., 2018; Lansdale, 2022; Luyando, 2018; Schwerdtner et al., 2017; Voorhees et al., 2006; Vuichard et al., 2011). 'a' corresponded to replicate one. Replicates 'b' and 'c' referred to the second and third sets of samples. For samples 8a, 8b, and 8c, no human semen was added because

these samples were used as negative controls to ensure no contamination was present in the reagents used.

Evidentiary Swab Extraction

The positive control was neat semen or the 1:1 dilution, while the negative control was a reagent blank. For the control group, no horse semen was added, while for the experimental group, 50µL of horse semen was added. Then, 395µL of e-cell digestion buffer and 5µL of proteinase K were added to each sample tube. After being vortexed for 30 seconds, the tubes were incubated at 56°C for 1 hour. Following the incubation, the samples were centrifuged in a short burst to remove the condensation from the tube lids. Next, the swabs and digestion solutions were transferred to new, sterile tubes labeled "sperm fraction" with spin baskets. After being transferred, the samples were centrifuged at 12,200 rotations per minute for 5 minutes. The swabs and spin baskets were removed. Without disturbing the sperm pellet, the supernatant was removed and placed in a new tube labeled "epithelial fraction." The epithelial fraction samples were stored in the -20°C freezer until phenol/chloroform/isoamyl alcohol extractions were performed. The sperm pellet was washed three separate times using Ultrapure Distilled water by 500µL of water to each sperm fraction sample. After vortexing for 10 seconds, the samples were centrifuged at 12,200 rotations per minute for 5 minutes. Once the tubes were centrifuged, the supernatant was removed without disturbing the sperm pellet and retained in pre-labeled tubes. After the three washing steps, the sperm fractions were lysed by adding 500µL of e-cell digestion buffer and 5µL of dithiothreitol. Next, the fractions were vortexed for 30 seconds, quickly centrifuged, and incubated at 70°C for 40 minutes. Finally, the samples were retained until phenol/chloroform/isoamyl alcohol extractions were performed (Oklahoma State Bureau of Investigation, 2023).

DNA Extraction and Purification

For this project, male DNA from the sperm fraction samples was extracted using phenol/chloroform/isoamyl alcohol and purified using ethanol precipitation. First, 225µL of stain extraction buffer and 5µL of proteinase K were added to the samples, which were then incubated at 55°C for 10 minutes before centrifuging. Next, 500 to 650 µL of phenol/chloroform/isoamyl alcohol was added, followed by vortexing for 2 seconds and centrifuging at room temperature for 60 seconds at maximum speed. The aqueous layer was removed and placed into a sterile 1.5mL microfuge tube. The original microfuge tubes were kept until experimentation was completed. To the removed aqueous layer, 22µL of 3M sodium acetate, pH 5.2, was added and inverted to mix. Next, 284µL of ice-cold 100% ethanol was added, followed by a 2-second vortex. The samples were then placed on ice for 30 minutes. After the icing, the samples were centrifuged for 10 minutes at maximum speed. After removing the supernatant and placing it into a pre-labeled tube, 1mL of 70% ethanol was added to each sample. Samples were inverted and centrifuged for 10 minutes at maximum speed. The supernatant was transferred into pre-labeled supernatant tubes, and the sperm pellet was air-dried at room temperature for 5 minutes. Finally, the pellet was redissolved in 50µL of TE[4] buffer and vortexed for 15 seconds at max speed. The extracted samples were stored at -20°C until further analysis (Lansdale, 2022).

Quantification

The purified samples were quantified using Applied Biosystems' Quantifiler™ HP kit and Applied Biosystems' QuantStudio™ 5 Real-Time PCR System for Human Identification. First, a DNA quantification standard dilution series was made based on Table 2.

Table 1. Quantifiler™ HP Standard Dilution Series Creation

Standard (Std.)	Concentration (ng/μL)	Final Conc. (ng/μL)
Std. 1	10μL [100ng/μL] + 10μL Quantifiler™ THP dilution buffer	50.00
Std. 2	10μL [Std. 1] + 90μL dilution buffer	5.000
Std. 3	10μL [Std. 2] + 90μL dilution buffer	0.500
Std. 4	10μL [Std. 3] + 90μL dilution buffer	0.050
Std. 5	10μL [Std. 4] + 90μL dilution buffer	0.005

Next, a PCR mix was made with 8μL of Quantifiler™ HP Primer Mix and 10μL of Quantifiler™ THP PCR Reaction Mix per sample. The mix was vortexed for 3 to 5 seconds prior to allocation. There were 48 samples in total. However, the quantification was done in triplicate. Therefore, there were 144 samples, along with 4 positive controls and 4 negative controls. This yielded a total number of 152 samples. A master mix was made by combining 1280μL of Primer Mix and 1600μL of PCR Reaction Mix in a sterile 2.5mL microfuge tube. After vortexing the master mix for 3 to 5 seconds, the mix was briefly centrifuged. Next, 18μL of the mix was added to 84 reaction wells of the two 96-well reaction plates needed for the experiment. For the negative controls, 2μL of amplification-grade water was added after the master mix. Then, 2μL of each experimental sample were added to the reaction mix for each of the test samples. Finally, 2μL of each standard dilution was added to the plate for the positive controls. The plate was sealed using MicroAmp® Optical Adhesive Film and centrifuged at 3000 rotations per minute for 20 seconds. After centrifugation, the plate was loaded into the QuantStudio™ 5 PCR System and was quantified under the following parameters: 95°C for 2 minutes and 40 cycles of 95°C for 9 seconds and 60°C for 30 seconds (Applied Biosystems, 2018).

Results

The standard curve for the control group followed the trendlines exactly and yielded R^2 values of 0.998 and 0.99 for large autosomal and small autosomal, respectively (Figure 1). Similarly, the R^2 values for the experimental group were 0.991 and 0.985 for large autosomal and small autosomal, respectively (Figure 2). The slopes for the control group were -3.433 and -3.1 for large and small autosomal (Figure 1). The slopes for the experimental group were -3.341 and -3.182 for large and small autosomal (Figure 2).

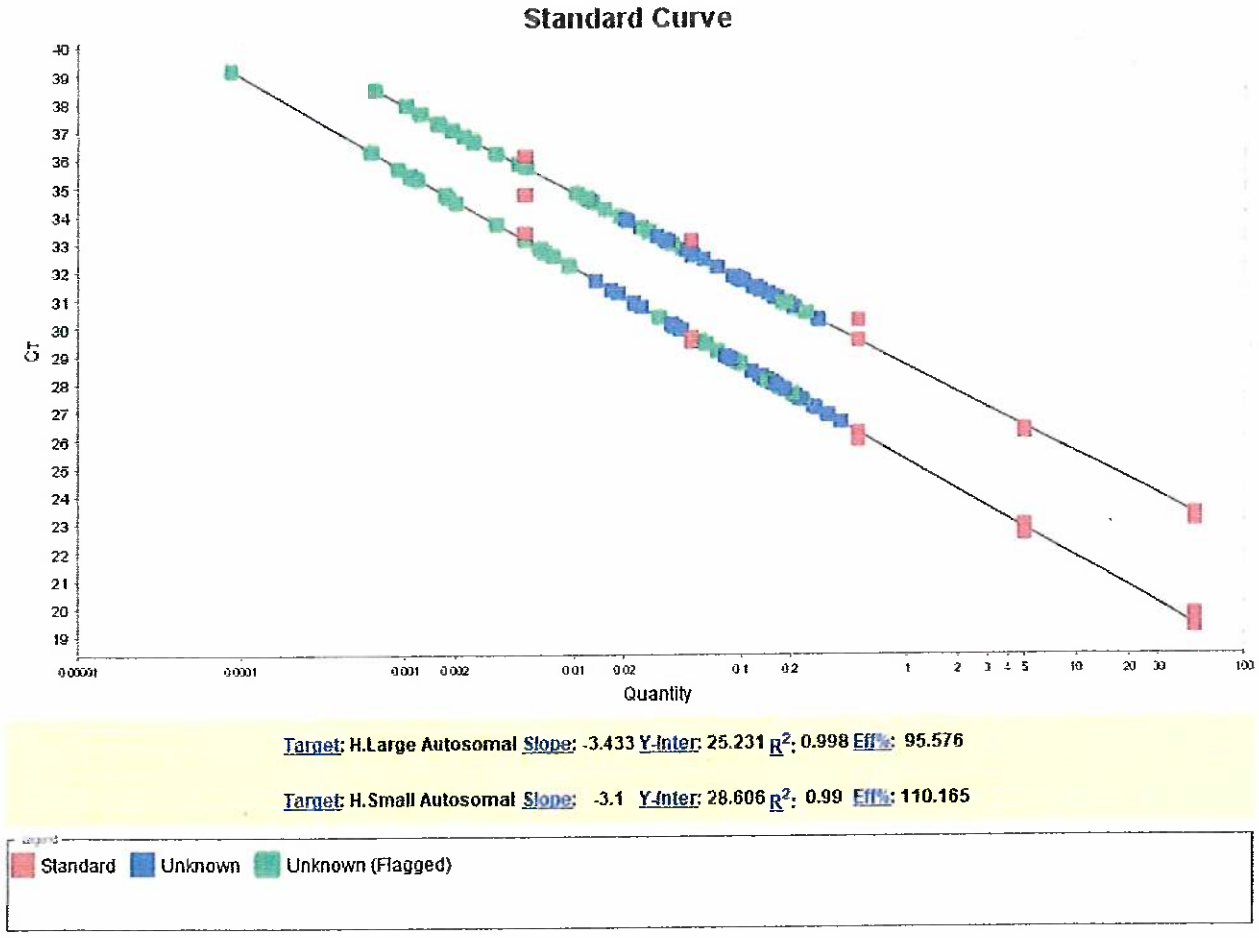

Figure 1. Quantifiler™ HP Control Group Standard Curves

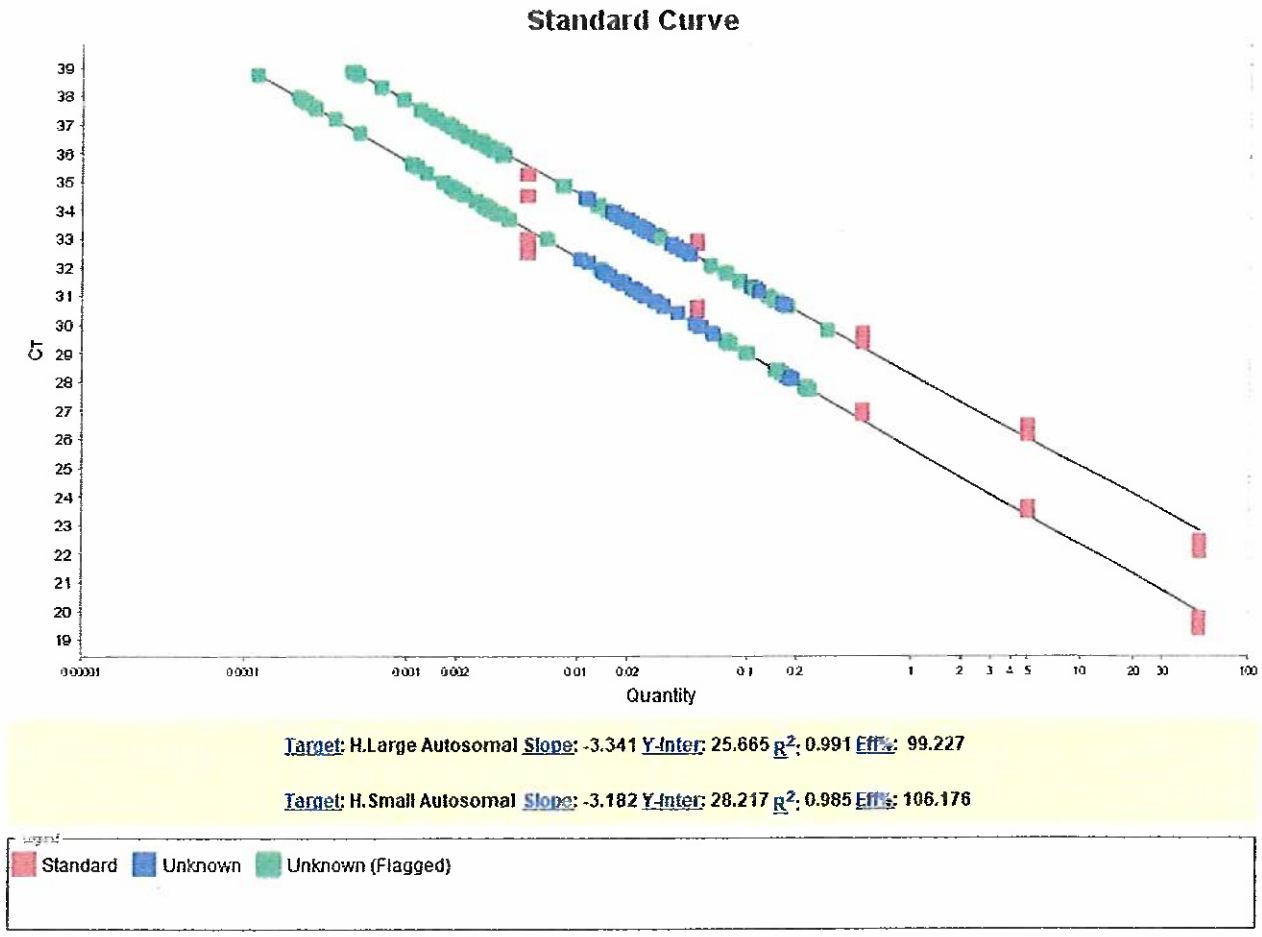

Figure 2. Quantifiler™ HP Experimental Group Standard Curves

Table 2 represents the Quantifiler™ HP large autosomal quantification data for the sperm fraction extracted from simulated sexual assault swabs from the control group that were created using semen dilutions and which did not have horse semen added. The average DNA concentrations were calculated for the sperm fraction triplicates (highlighted light blue) and for the sample replicates (highlighted light green) (Table 2).

Table 2. Large Autosomal Quantification Results for the Control Group without Horse Semen

	Replicate a	Replicate b	Replicate c		
Dilution	*DNA Conc. (ng/μL)*	*DNA Conc. (ng/μL)*	*DNA Conc. (ng/μL)*	*Avg. DNA Conc. (ng/μL)*	*Standard Deviation*
1:1	0.1831	0.1514	0.3248	0.2198	0.0923
1:1	0.1638	0.1452	0.3347	0.2146	0.1045
1:1	0.1569	0.1422	0.3942	0.2311	0.1414
Avg.	0.1680	0.1463	0.3512	0.2218	0.1126
1:2	0.1747	0.1523	0.3313	0.2194	0.0975
1:2	0.1431	0.2038	0.2827	0.2099	0.0700
1:2	0.1444	0.1726	0.2719	0.1963	0.0670
Avg.	0.1541	0.1762	0.2953	0.2085	0.0760
1:4	0.1287	0.1719	0.1150	0.1385	0.0300
1:4	0.1503	0.2261	0.1325	0.1696	0.0497
1:4	0.1546	0.2122	0.1377	0.1682	0.0391
Avg.	0.1445	0.2034	0.1284	0.1588	0.0395
1:8	0.0826	0.2104	0.0562	0.1164	0.0825
1:8	0.0801	0.2237	0.0611	0.1216	0.0889
1:8	0.0870	0.2387	0.0722	0.1326	0.0921
Avg.	0.0832	0.2243	0.0632	0.1236	0.0878
1:16	0.0946	0.0432	0.0421	0.0600	0.0300
1:16	0.0984	0.0435	0.0321	0.0580	0.0354
1:16	0.0910	0.0397	0.0327	0.0545	0.0318
Avg.	0.0947	0.0421	0.0356	0.0575	0.0324
1:32	0.0402	0.0227	0.0074	0.0234	0.0164
1:32	0.0387	0.0023	0.0091	0.0167	0.0194
1:32	0.0379	0.0250	0.0177	0.0269	0.0102
Avg.	0.0389	0.0167	0.0110	0.0223	0.0146
1:64	0.0049	0.0132	0.0034	0.0072	0.0053
1:64	0.0062	0.0181	0.0001	0.0081	0.0092
1:64	0.0065	0.0164	0.0006	0.0078	0.0080
Avg.	0.0059	0.0159	0.0014	0.0077	0.0074
1:0*	0.0011	0.0012	0.0017	0.0013	0.0003
1:0*	0.0011	0.0011	0.0018	0.0013	0.0004
1:0*	0.0006	0.0009	0.0020	0.0012	0.0007
Avg.	0.0009	0.0011	0.0018	0.0013	0.0005

Table 3 shows the large autosomal quantification data for the experimental group where horse semen was added prior to extraction. The average DNA concentrations for the experimental group were calculated in the same manner as the control group (Table 3).

Table 3. Large Autosomal Quantification Results for the Experimental Group with Horse Semen

	Replicate a	Replicate b	Replicate c		
Dilution	*DNA Conc. (ng/µL)*	*DNA Conc. (ng/µL)*	*DNA Conc. (ng/µL)*	*Avg. DNA Conc. (ng/µL)*	*Standard Deviation*
1:1	0.0635	0.0321	0.0248	0.0401	0.0206
1:1	0.0615	0.0383	0.0208	0.0402	0.0204
1:1	0.0630	0.0319	0.0217	0.0389	0.0215
Avg.	0.0627	0.0341	0.0224	0.0397	0.0207
1:2	0.1855	0.0231	0.2261	0.1449	0.1074
1:2	0.1875	0.0207	0.2226	0.1436	0.1078
1:2	0.1790	0.0184	0.2394	0.1456	0.1142
Avg.	0.1840	0.0207	0.2290	0.1447	0.1097
1:4	0.0492	0.0143	0.1490	0.0708	0.0699
1:4	0.0524	0.0153	0.0750	0.0476	0.0301
1:4	0.0535	0.0172	0.1613	0.0773	0.0749
Avg.	0.0517	0.0156	0.1284	0.0652	0.0576
1:8	0.2289	0.0183	0.1515	0.1329	0.1065
1:8	0.0793	0.0211	0.1718	0.0907	0.0760
1:8	0.1000	0.0177	0.1617	0.0931	0.0722
Avg.	0.1361	0.0190	0.1617	0.1056	0.0760
1:16	0.0281	0.0035	0.0029	0.0115	0.0144
1:16	0.0290	0.0031	0.0039	0.0120	0.0147
1:16	0.0238	0.0020	0.0034	0.0100	0.0122
Avg.	0.0270	0.0029	0.0034	0.0111	0.0138
1:32	0.0137	0.0013	0.0131	0.0094	0.0070
1:32	0.0101	0.0010	0.0138	0.0083	0.0066
1:32	0.0113	0.0011	0.0998	0.0374	0.0543
Avg.	0.0117	0.0011	0.0422	0.0184	0.0213
1:64	0.0001	0.0022	0.0065	0.0029	0.0033
1:64	0.0011	0.0026	0.0018	0.0018	0.0008
1:64	0.0016	0.0029	0.0019	0.0021	0.0007
Avg.	0.0009	0.0026	0.0034	0.0023	0.0013
1:0*	N/Q	0.0005	0.0003	0.0004	0.0001
1:0*	0.0003	0.0004	0.0002	0.0003	0.0001
1:0*	N/Q	0.0002	0.0002	0.0002	0.0000
Avg.	0.0003	0.0004	0.0002	0.0003	0.00007

Table 4 represents the Quantifiler™ HP small autosomal quantitation data for the sperm fraction extracted from simulated sexual assault swabs from the control group that were created using semen dilutions. The average DNA concentrations were calculated for the sperm fraction

triplicates (highlighted light blue) and for the sample replicates (highlighted light green), as seen in Table 4.

Table 4. Small Autosomal Quantification Results for the Control Group without Horse Semen

	Replicate a	**Replicate b**	**Replicate c**		
Dilution	*DNA Conc. (ng/µL)*	*DNA Conc. (ng/µL)*	*DNA Conc. (ng/µL)*	*Avg. DNA Conc. (ng/µL)*	*Standard Deviation*
1:1	0.0898	0.1008	0.2096	0.1334	0.0662
1:1	0.0955	0.0979	0.1557	0.1164	0.0341
1:1	0.1294	0.1036	0.1915	0.1415	0.0452
Avg.	0.1049	0.1008	0.1856	0.1304	0.0479
1:2	0.095	0.0388	0.2879	0.1406	0.1307
1:2	0.1808	0.1779	0.1818	0.1802	0.0020
1:2	0.1886	0.2452	0.2952	0.243	0.5333
Avg.	0.1548	0.1540	0.2550	0.1879	0.0581
1:4	0.144	0.1637	0.1591	0.1556	0.0103
1:4	0.1226	0.2037	0.186	0.1708	0.0426
1:4	0.104	0.1631	0.1221	0.1297	0.0303
Avg.	0.1235	0.1768	0.1557	0.1520	0.0268
1:8	0.0717	0.1237	0.0278	0.0744	0.0480
1:8	0.0511	0.0972	0.0188	0.0557	0.0394
1:8	0.0361	0.2154	0.0441	0.0985	0.1013
Avg.	0.0530	0.1454	0.0302	0.0762	0.0610
1:16	0.0524	0.0363	0.0309	0.0399	0.0112
1:16	0.0251	0.0344	0.0126	0.0240	0.0109
1:16	0.0704	0.0459	0.0356	0.0506	0.0179
Avg.	0.0493	0.0389	0.0264	0.0382	0.0115
1:32	0.0355	0.0207	0.0151	0.0238	0.0105
1:32	0.0463	0.0249	0.0126	0.0279	0.0171
1:32	0.0592	0.0314	0.0363	0.0423	0.0148
Avg.	0.047	0.0257	0.0213	0.0313	0.0137
1:64	0.0047	0.0206	0.0051	0.0101	0.0091
1:64	0.0103	0.0198	0.0006	0.0102	0.0096
1:64	0.0116	0.012	0.0010	0.0082	0.0062
Avg.	0.0089	0.0175	0.0022	0.0095	0.0076
1:0*	0.0012	0.0016	0.0026	0.0018	0.0007
1:0*	0.0016	0.0019	0.0034	0.0023	0.0010
1:0*	0.0016	0.0022	0.0025	0.0021	0.0005
Avg.	0.0015	0.0019	0.0028	0.0021	0.0007

Table 5 outlines the Quantifiler™ HP small autosomal quantitation data for the sperm fraction extracted from simulated sexual assault swabs from the experimental group that were created using semen dilutions. Horse semen was added before the first e-cell digestion. The average DNA concentrations were calculated for the sperm fraction triplicates (highlighted light blue) and for the sample replicates (highlighted light green) as shown in Table 5. An asterisk was used to indicate the negative controls used to determine if there was contamination in the purified water. The N/Q represented samples that were not quantified.

Table 5. Small Autosomal Quantification Results for the Experimental Group with Horse Semen

	Replicate a	Replicate b	Replicate c		
Dilution	*DNA Conc. (ng/μL)*	*DNA Conc. (ng/μL)*	*DNA Conc. (ng/μL)*	*Avg. DNA Conc. (ng/μL)*	*Standard Deviation*
1:1	0.0459	0.0277	0.1504	0.0747	0.0541
1:1	0.0354	0.0294	0.1758	0.0802	0.0676
1:1	0.0408	0.0243	0.3066	0.1239	0.1294
Avg.	0.0407	0.0271	0.2109	0.0929	0.0836
1:2	0.1169	0.0209	0.0226	0.0535	0.0449
1:2	0.1617	0.0245	0.0231	0.0698	0.0650
1:2	0.1700	0.0233	0.0201	0.0711	0.0699
Avg.	0.1495	0.0229	0.0219	0.0648	0.0599
1:4	0.0380	0.0109	0.1334	0.0608	0.0525
1:4	0.0437	0.0158	0.0599	0.0398	0.0182
1:4	0.0456	0.0179	0.1098	0.0578	0.0385
Avg.	0.0424	0.0149	0.1010	0.0528	0.0359
1:8	0.1477	0.0450	0.1060	0.0996	0.0422
1:8	0.0307	0.0131	0.1111	0.0516	0.0427
1:8	0.0748	0.0159	0.1055	0.0654	0.0372
Avg.	0.0844	0.0247	0.1075	0.0722	0.0349
1:16	0.0254	0.0012	0.0036	0.0101	0.0109
1:16	0.0192	0.0033	0.0030	0.0085	0.0076
1:16	0.0226	0.0015	0.0023	0.0088	0.0098
Avg.	0.0224	0.0020	0.0030	0.0091	0.0094
1:32	0.0168	0.0019	0.0113	0.0100	0.0062
1:32	0.0159	0.0020	0.0148	0.0109	0.0063
1:32	0.0115	0.0021	0.0907	0.0348	0.0397
Avg.	0.0147	0.0020	0.0389	0.0186	0.0153
1:64	0.0017	0.0026	0.0082	0.0042	0.0029
1:64	0.0013	0.0027	0.0026	0.0022	0.0006
1:64	0.0023	0.0020	0.0023	0.0022	0.0001
Avg.	0.0018	0.0024	0.0044	0.0030	0.0011
1:0*	0.0005	0.0030	0.0005	0.0013	0.0012
1:0*	0.0007	N/Q	0.0036	0.0022	0.0015
1:0*	0.0009	N/Q	N/Q	0.0009	0.0000
Avg.	0.0007	0.0030	0.0021	0.0020	0.0009

Additionally, the percentage differences were calculated based on the average DNA concentrations from the control group without horse semen added and were calculated for both large and small autosomal (Table 6). The values colored dark red in Table 6 represent positive percentage differences. The standard deviations for the large and small autosomal have been calculated for

the control and experimental groups (Tables 2, 3, 4, and 5). The average standard deviation between the sperm fraction triplicates was highlighted in light blue.

Table 6. Percent Differences Between the Large and Small Autosomal Average DNA Yields with and without Horse Semen

Dilution	% Diff. Large Control vs. Large Experimental	% Diff. Small Group vs. Small Experimental
1:1	-81.74	-44.03
1:1	-81.26	-31.09
1:1	-83.18	-12.44
Avg.	-82.09	-28.75
1:2	-33.97	-61.96
1:2	-31.58	-61.28
1:2	-25.83	-70.73
Avg.	-30.61	-65.52
1:4	-48.87	-60.95
1:4	-71.96	-76.69
1:4	-54.01	-55.47
Avg.	-58.91	-65.29
1:8	14.18	33.83
1:8	-25.40	-7.30
1:8	-29.78	-33.63
Avg.	-14.54	-5.26
1:16	-80.82	-74.75
1:16	-79.31	-64.63
1:16	-82.13	-82.62
Avg.	-80.73	-76.11
1:32	-60.03	-57.92
1:32	-50.25	-60.98
1:32	39.21	-17.81
Avg.	-17.79	-40.78
1:64	-59.07	-58.88
1:64	-77.46	-78.50
1:64	-72.77	-73.17
Avg.	-70.17	-70.01
1:0*	-70.00	-25.93
1:0*	-77.50	-6.52
1:0*	-82.86	-57.14
Avg.	-76.52	-7.26

Finally, the degradation index was calculated for the control and experimental groups by dividing the small autosomal DNA concentration by the large autosomal DNA concentration (Table 7). The

averages between sperm fractions were highlighted in light blue. Additionally, the blue font color was used to indicate which indices were between 1 and 10.

Table 7. Degradation Indices for the Control and Experimental Groups

Dilution:	**Degradation Index: (Control Small Autosomal/ Control Large Autosomal)**	**Degradation Index: (Experimental Small Autosomal/Experimental Large Autosomal)**
1:1	0.6070	1.8605
1:1	0.5423	1.9950
1:1	0.6123	3.1878
Avg.	0.5880	2.3386
1:2	0.6406	0.3690
1:2	0.8585	0.4858
1:2	1.2379	0.4886
Avg.	0.9011	0.4477
1:4	1.1231	0.8579
1:4	1.0067	0.8367
1:4	0.7715	0.7470
Avg.	0.9575	0.8089
1:8	0.6318	0.7492
1:8	0.4579	0.5691
1:8	0.7429	0.7022
Avg.	0.6168	0.6838
1:16	0.6648	0.8754
1:16	0.4144	0.7083
1:16	0.9296	0.9041
Avg.	0.6642	0.8235
1:32	1.0142	1.0676
1:32	1.6743	1.3133
1:32	1.5744	0.9296
Avg.	1.4033	1.0109
1:64	1.4140	1.4205
1:64	1.2582	1.2000
1:64	1.0468	1.0313
Avg.	1.2349	1.2415
1:0*	1.3500	3.3333
1:0*	1.7250	7.1667
1:0*	1.8000	4.5000
Avg.	1.6174	6.3889

Figure 3 demonstrates how the average large autosomal DNA concentrations changed with each water:semen dilution for the control and experimental groups.

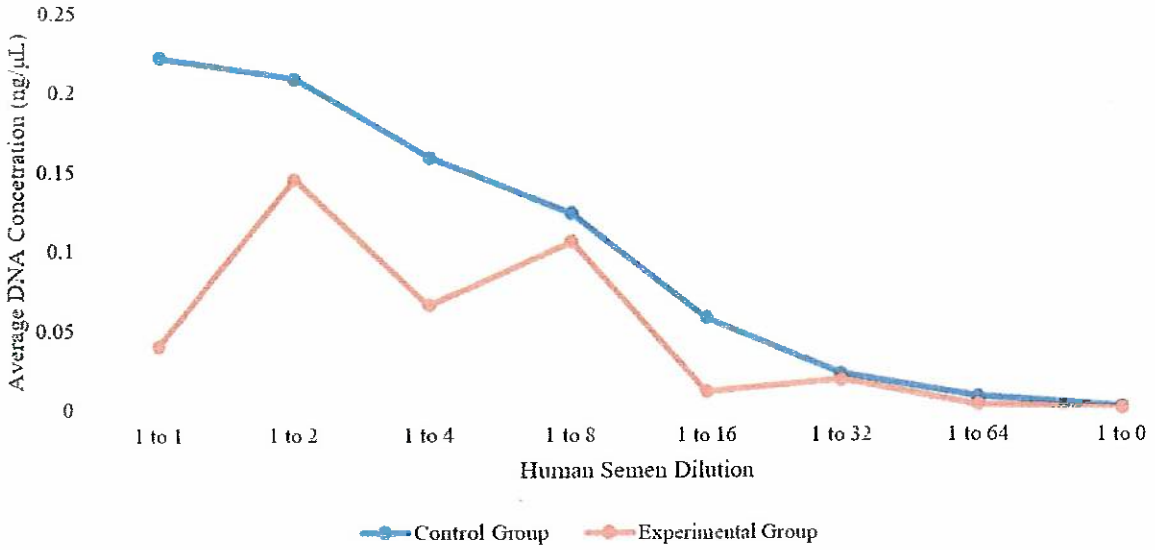

Figure 3. Comparison of the Average Large Autosomal Human DNA Yield with and without Horse Semen

Figure 4 demonstrates the changes in the average small autosomal DNA concentrations for both groups.

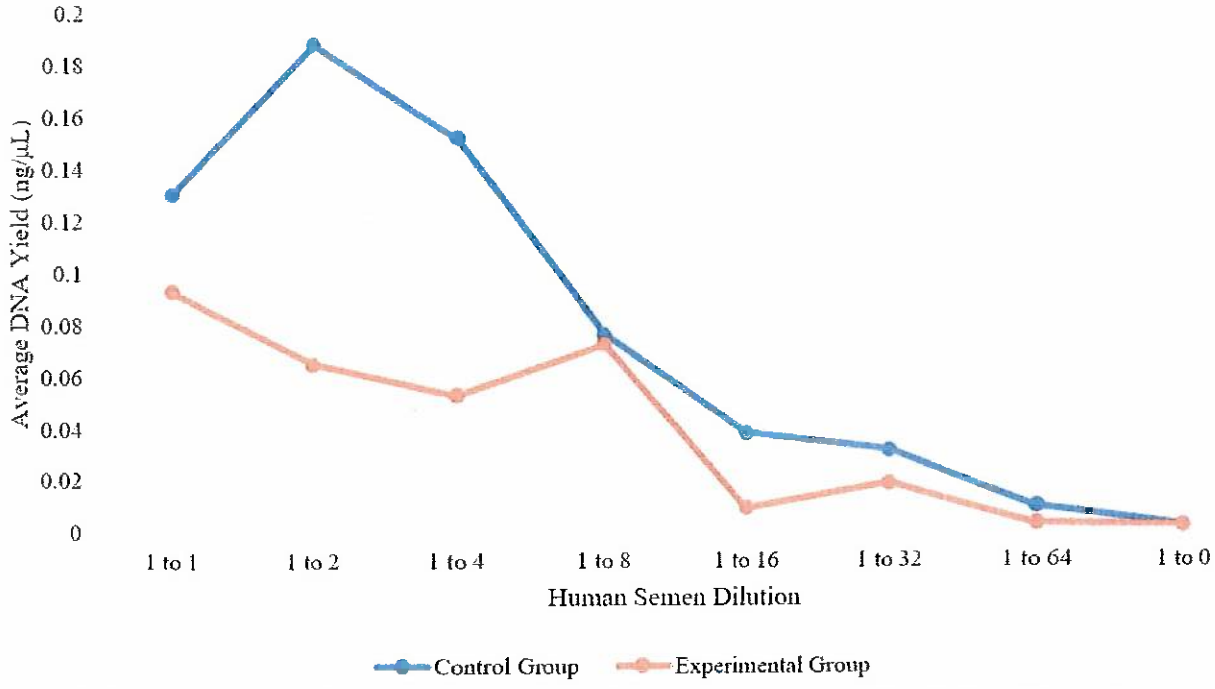

Figure 4. Comparison of the Average Small Autosomal Human DNA Yield with and without Horse Semen

Figure 5 illustrated the comparison between the average degradation indices for the control and experimental groups. In this project, horse semen was added to the experimental group only.

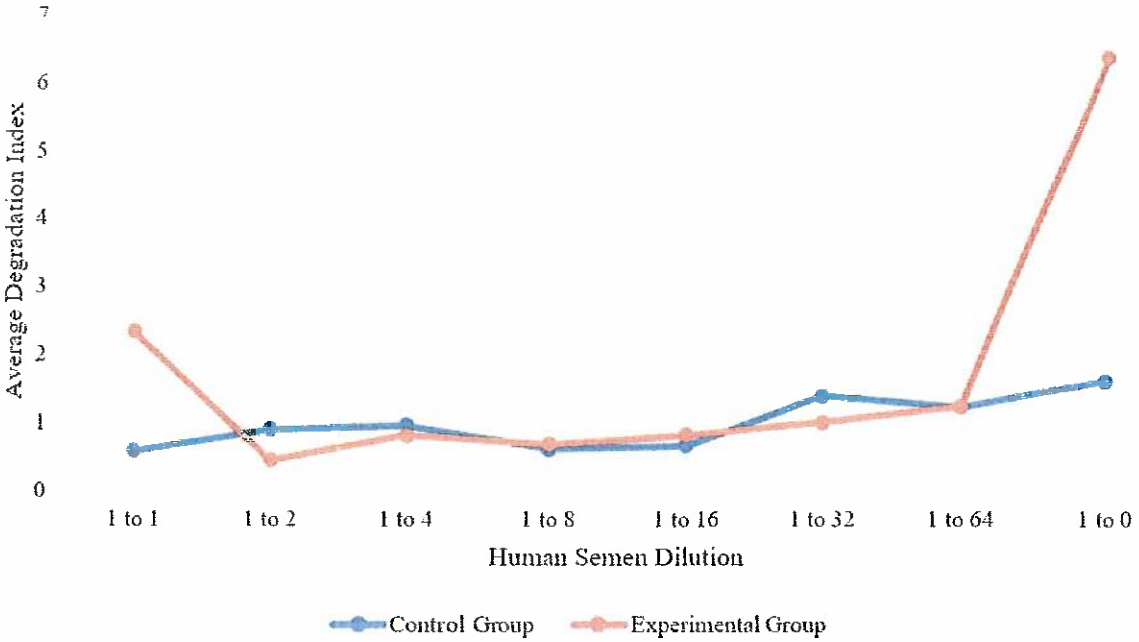

Figure 5. Comparison of the Average Degradation Indices with and without Horse Semen

Figure 6 compares the average DNA concentrations between the control and experimental groups for the large autosomal to provide a visual demonstration of the differences between the two groups. The numbers 1 through 8 on the x-axis represent the water:human semen dilutions 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:0, respectively.

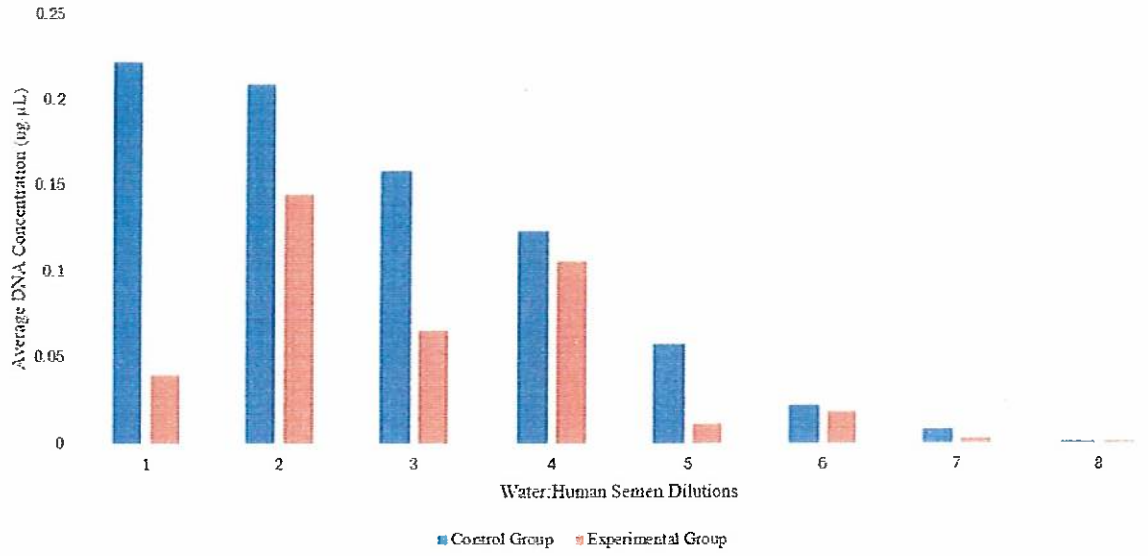

Figure 6. Average Large Autosomal Human DNA Concentrations for the Control and Experimental Groups

Figure 7 compares the average DNA concentrations between the control and experimental groups for the small autosomal to provide a visual demonstration of the differences between the two groups. The numbers 1 through 8 on the x-axis represent the water:human semen dilutions 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:0, respectively.

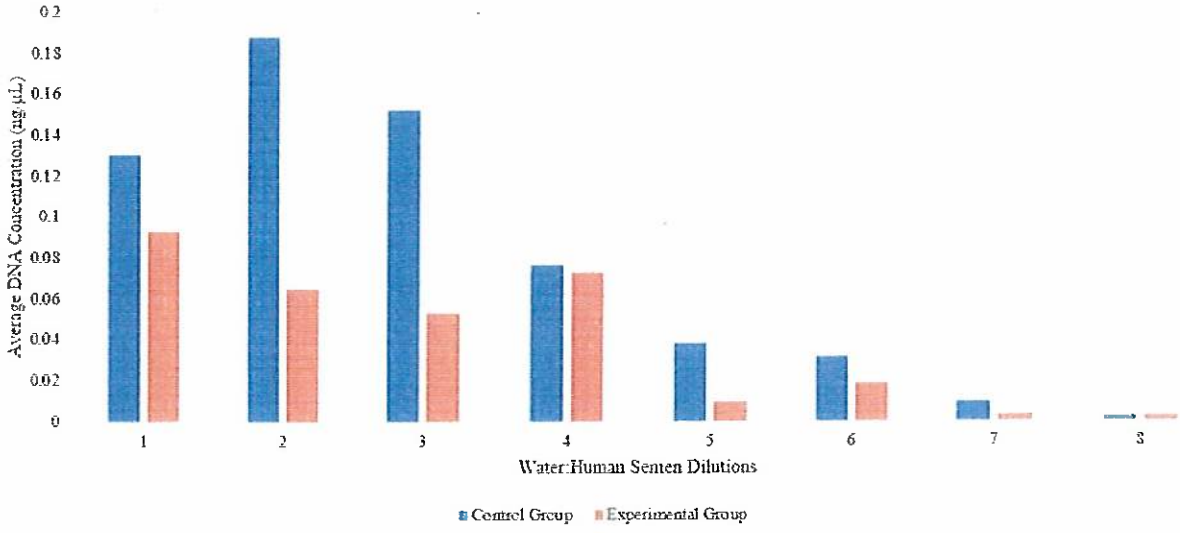

Figure 7. Average Small Autosomal Human DNA Concentrations for the Control and Experimental Groups

Discussion

According to Applied Biosystems™ (2018), an R^2 greater than or equal 0.99 demonstrates a tight fit between the standard curve regression line and the individual cycle threshold data points. The R^2 values for the control group are greater than 0.99, which is indicative of a proper fit. One of the R^2 values for the experimental group is greater than 0.99, while the small autosomal value is 0.985. Because 0.985 is less than 0.99, the fit for the small autosomal experimental group is not considered tight. The slope range for the large autosomal and small autosomal should fall between -3.1 to -3.7 and -3.0 to -3.6, respectively, which can be observed in Figures 1 and 2. Since the standard curves met the manufacturer's guidelines, the Quantifiler™ HP kit was correctly used; therefore, any failed quantifications were not caused by the kit.

For the large autosomal, human-only group, the average DNA concentrations can be found in Table 2. The average concentrations decreased with increasing dilution. This trend was expected because as the dilution increases, the concentration of sperm cells decreases. The average standard deviations for this group are listed in Table 2—these values indicate that the sperm fraction replicates (a, b, and c) yielded concentrations that remained close to the mean (Table 2). For the large autosomal, human-horse group, the DNA concentrations can be found in Table 3. The concentrations decreased with increasing dilution, except for the 1:2, 1:8, and 1:32 dilutions. One reason for this disparity is human error when creating the dilution series due to cross-contamination between the dilutions. The experimental group's average standard deviations are listed in Table 3. While a 10:1 carrier-to-DNA ratio described by Shaw et al. (2009) was not created during this project, the 1:4 and 1:8 water:semen dilutions were the closest by having a 12.5:1 carrier semen:human semen and a 6.25:1 carrier semen:human semen ratio, respectively. The 1:4 dilution recovered ~41% of the human DNA when horse semen was added (Figure 6 and

7). This percentage was calculated by dividing the 1:4 dilution DNA concentration from the experimental group by the 1:4 dilution DNA concentration from the control group. The 1:8 dilution recovered ~85% (Figure 6 and 7).

The average DNA concentrations for the small autosomal, human-only group can be found in Table 4. The average concentrations decreased with increasing dilution, except for the 1:1 dilution (Table 4). This trend was expected because as the dilution increases, the concentration of sperm cells decreases. The most likely reason the 1:1 dilution had a lower average DNA concentration is human error during the dilution creation process. The average standard deviations for the control group can be found in Table 4—as mentioned above with the large autosomal targets, standard deviations less than the average indicated the data are close to the mean. The average DNA concentrations for the small autosomal, human-horse group can be found in Table 5. The average concentration decreased with increasing dilution, except for the 1:32 dilution (Table 5). This trend is expected because as the dilution increases, the concentration of sperm cells decreases. The most likely reason the 1:32 dilution had a higher average DNA concentration was human error during the dilution creation process. The average standard deviations are shown in Table 5. While a 10:1 carrier-to-DNA ratio described by Shaw et al. (2009) was not created during this project, the 1:4 and 1:8 water:semen dilutions were the closest by having a 12.5:1 carrier semen:human semen and a 6.25:1 carrier semen:human semen ratio, respectively. The 1:4 dilution recovered ~35% of the human DNA when horse semen was added (Figure 6 and 7). This percentage was calculated by dividing the 1:4 dilution DNA concentration from the experimental group by the 1:4 dilution DNA concentration from the control group. The 1:8 dilution recovered ~95% (Figure 6 and 7).

The percentage differences were calculated for the large autosomal experimental group with horse semen. All differences were negative except for the 1:8 and 1:32 dilutions (14.18% and 39.21%, respectively). The positive values indicated that there was more DNA in the experimental group than the control group. However, the more likely scenario that caused the increase would be carryover from the epithelial fraction into the sperm fraction. Garvin et al. (2012) estimated that there can be 0.0048% to 20% female DNA carryover into the sperm fraction. The average percentage differences for the large autosomal fragment can be found in Table 6. The overall large autosomal average percentage difference between the control and experimental groups was -53.92%. The percentage differences were calculated for the small autosomal experimental group with horse semen. All values were negative except for one of the 1:8 dilution replicates, which had a positive value (33.83%). This outliner could be due to contamination from the epithelial fraction into the sperm fraction for this sample or because sperm cell DNA was extracted more efficiently. The small autosomal average percentage differences are listed in Table 6. The overall average percentage difference was -44.87%. This percentage difference was smaller than what Lansdale (2022) reported (~99%). The drastic difference between the percent values can be attributed to the fact that Lansdale (2022) did not use simulated sexual assault samples, leading to higher DNA yields.

In addition to the average percentage differences and standard deviations, the degradation indices were calculated for each dilution for the control and experimental groups to determine if any DNA was degraded. The degradation index was calculated by dividing the small autosomal DNA concentration by the large autosomal DNA concentration (Table 7). The average degradation indices for the control group for each dilution can be found in Table 7. Because the concentration of small and large autosomal fragments is approximately equal, intact DNA will have a

degradation index of less than or equal to 1—a degradation index higher than 1 could mean the DNA is degraded (Lackey, 2018). Therefore, the 1:32, 1:64, and 1:0 dilutions could contain degraded DNA. However, those dilutions had low starting concentrations of sperm cells, meaning the higher indices could be the result of the lower concentrations. The average degradation indices for the experimental group for each dilution can be found in Table 7. For the experimental group, the 1:1, 1:32, 1:64, and 1:0 dilutions had degradation indices greater than 1, which could demonstrate DNA degradation. Figure 5 provides a comparison between the experimental and control groups. The control group demonstrated more uniform data, while the experimental group showed two outliers (Figure 5). The uniformity of the control group may have been due to the higher DNA concentrations in the dilutions. In contrast, the experimental group's outliers may have been due to low starting DNA concentrations.

Figures 6 and 7 demonstrate the drastic recovery differences between the control and experimental groups for both large and small autosomal. Our hypothesis is that using the horse semen can led to a higher recovery rate of human male DNA. However, both the large and small autosomal experimental groups showed decreased human male DNA yields. In comparison, water:human semen dilutions 1:8 and 1:32 in the experimental group recovered approximately 95% and 59% human male DNA, respectively. Neither dilution corresponded to a horse semen:human semen ratio of 10:1—the 1:8 water:human semen dilution had a horse semen:human semen ratio of 6.25:1 while the 1:32 dilution had a horse:human ratio of 1.5625:1.

Despite the addition of horse semen, the experimental group (orange line) showed a higher loss of human semen compared with the control group (blue line) for both the large and small autosomal fragments (Figures 3 and 4). Based on the above percents, the hypothesis that using mammalian sperm as a carrier was refuted because there was a more significant loss of human

DNA in the group with horse semen than in the group without horse semen (Figures 3 and 4). As expected, both showed a decrease in sperm cells following an increase in dilution. DNA was found in the 1:0 dilutions for both groups. The average DNA concentrations for the 1:0 dilutions were 0.0013ng/μL, 0.0003ng/μL, 0.0021ng/μL, and 0.0020ng/μL for the large autosomal-control group, large autosomal-experimental group, small autosomal-control group, and small autosomal-experimental group, respectively (Tables 2, 3, 4, and 5). This indicated a negligible amount of contamination within the purified water or contamination introduced at some point during the experiment. The DNA concentrations for both groups were deficient, which could be due to the loss of sperm cells during differential extraction and phenol/chloroform/isoamyl alcohol extractions. Because there were two to three washing steps in both extraction procedures, a portion of sperm cells could have been lost due to the sperm pellet not being tightly packed. However, the addition of horse semen should have protected the sperm pellet during centrifugation. Based on the overall percentage differences for large and small autosomal fragments (-53.92% and -44.87%), the DNA concentrations were not increased by adding horse semen. Another reason for a lower concentration of sperm cells could be due to the sample substrate from which the sperm cell DNA was extracted. Some of the sperm cells may not have been successfully extracted from the buccal swabs, which would have contributed to the lower concentration of DNA. Finally, the stochastic threshold for this project was set to 0.2. Because the DNA concentrations were low, the threshold could have been too high, preventing an accurate depiction of the amounts of DNA within the sperm fractions. Based on the DNA concentration yields of both groups, this research confirmed that using a carrier sperm to retain human sperm cells was not a successful method.

Conclusions

For thirty-nine years, numerous research projects have been devoted to developing differential extraction protocols to retain as many sperm cells as possible while efficiently removing epithelial cells. In the 1980s, Gill et al. developed the first differential extraction method, preferential lysis, to separate epithelial DNA from sperm cells. In the 1990s, two research groups modified Gill et al.'s (1985) method by using milder reagents during the lysing step and by performing an extra washing step to ensure that epithelial cells had been removed (Wiegand et al., 1992; Yoshida et al., 1995). Additionally, Chen et al. (1998) developed a filtration-based method to separate sperm cells from epithelial cells based on physiological differences in size and shape. In the 2000s, researchers explored several avenues to increase the male DNA yield, such as antibody/antigen-based capture, robotic-based purification, laser capture microdissection, Differex™ System, microfabricated devices, microchip-based separation, enzymatic digestion, Nanotraps, dielectrophoretic separation, and carrier RNA (Greenspoon & Ban, 2002; Elliott et al., 2003; Tereba et al., 2004; Horsman et al., 2005; Voorhees et al., 2006; Valgreen & Edenberger, 2008; Shaw et al., 2009). Continuing the trend of automation and antibody/antigen capture, the 2010s also introduced optical-based trapping methods and fluorescence-based cell sorting (Chakrabarty, 2010; Vuichard et al., 2011; Pereira et al., 2012; Hulme et al., 2013; Li et al., 2014; Grojean & Castella, 2015; Xu et al., 2016; Tobe et al., 2017; Williamson et al., 2018; Auka et al., 2019). Finally, the 2020s contained projects that automated differential extraction, utilized bead-based separation, carrier sperm methods, DNA/RNA co-extractions, Nanofiber mesh separation, and direct-to-amplification sperm lysis methods (Smith, 2020; Sun et al., 2021; Lansdale, 2022; Woolf et al., 2023). Despite the numerous decades and research projects, forensic laboratories continue to use conventional differential extractions even though there are several improved

protocols that could be implemented instead. The reasoning behind this is that perfection is expected due to the sensitive nature of accurate forensics. Because results from DNA extractions are taken to a courtroom and presented to a juror, any methodology employed is required to be incredibly effective, efficient, accurate, and infallible. To this day, this kind of perfection remains unachievable.

The previously mentioned projects that used polyadenylic acid inspired using a carrier to improve the recovery of sperm DNA. Kishore et al. (2006) utilized polyadenylic acid to purify DNA from biological stains using robotic extraction techniques. Compared to traditional organic extraction techniques, robotic extractions yielded low DNA concentrations; however, when polyadenylic acid was added after cell lysis, the DNA yield was increased 20-fold (Kishore et al., 2006). Further research into the polyadenylic acid carrier successfully extracted DNA from buccal swabs using microfluidic silica monoliths (Shaw et al., 2009). Shaw et al. (2009) discovered that adding polyadenylic acid to a microfluidic device increased the DNA yield 5-fold. While these projects demonstrate applicability in a forensic setting, forensic laboratories do not use robotic techniques or microfabricated silica monoliths in casework. Lansdale (2022) utilized this carrier concept to extract male DNA from semen samples. Instead of polyadenylic acid, they used horse or salmon semen as the carrier, with the horse sperm cells added before the lysis buffer and the salmon sperm DNA added during the washing steps. Adding horse sperm cells or salmon sperm DNA resulted in a percentage decrease in human DNA of 99.45% and 98.36%, respectively when extracted with a DNA IQ™ kit. This was confirmed with a secondary study using phenol/chloroform extractions, which yielded a percentage loss of 88.40% and 84.25% for horse sperm cells and salmon sperm DNA, respectively (Lansdale, 2022). However, it is important to note that neither of the above research projects extracted human DNA from sexual assault-type

samples. Therefore, we sought to repeat the carrier sperm method using simulated sexual assault samples with horse semen being added prior to extraction. The simulated samples were extracted using the Oklahoma State Bureau of Investigation's differential extraction protocol followed by purification via phenol/chloroform/isoamyl alcohol extractions with ethanol precipitation.

With the addition of horse sperm, there was a 44.87% decrease for the small autosomal and a 53.92% decrease for the large autosomal in sperm DNA compared to the control group (Table 6). Seven of the water:semen dilutions had degradation indices greater than 1, indicating potential DNA degradation. Based on the above values, the hypothesis that using a carrier sperm could be used to increase the yield of human sperm cells from simulated sexual assault samples was refuted. The results of this thesis confirmed Lansdale's (2022) findings.

Improving the differential extraction process is vital to eliminate the sexual assault kit backlog and bring justice to the victims of sexual assault crimes. In addition, DNA analysis should be streamlined, relatively fast, non-laborious, and cheap. This thesis aimed to develop a methodology that would increase the male DNA yield during differential extractions, which could be applied to automated systems in the future. However, the research conducted during this thesis validated Lansdale's (2022)—finding that the addition of mammalian sperm drastically decreased the human male DNA yield by 44%-99%. Typically, sexual assault evidence has an overabundance of female DNA and an underabundance of male DNA. Therefore, losing further male DNA from already diminished amounts directly impacts the STR profiles that are created during genetic analysis. The generated STR profiles using low DNA yields are impossible to analyze and compare to reference profiles. Simply put, the idea of a mammalian DNA carrier does not work within forensic DNA analysis. Therefore, the postulate of a mammalian carrier sperm should be abandoned for the foreseeable future. Future research should focus on improving differential

extraction through other means such as optical-based trapping or microfluidic devices, which have consistently increased the male DNA yield.

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Appendix A: Quantification Reports Generated for the Control Group and the Experimental Group

Control Group: Pages 79 – 102

Experimental Group: Pages 103 – 126

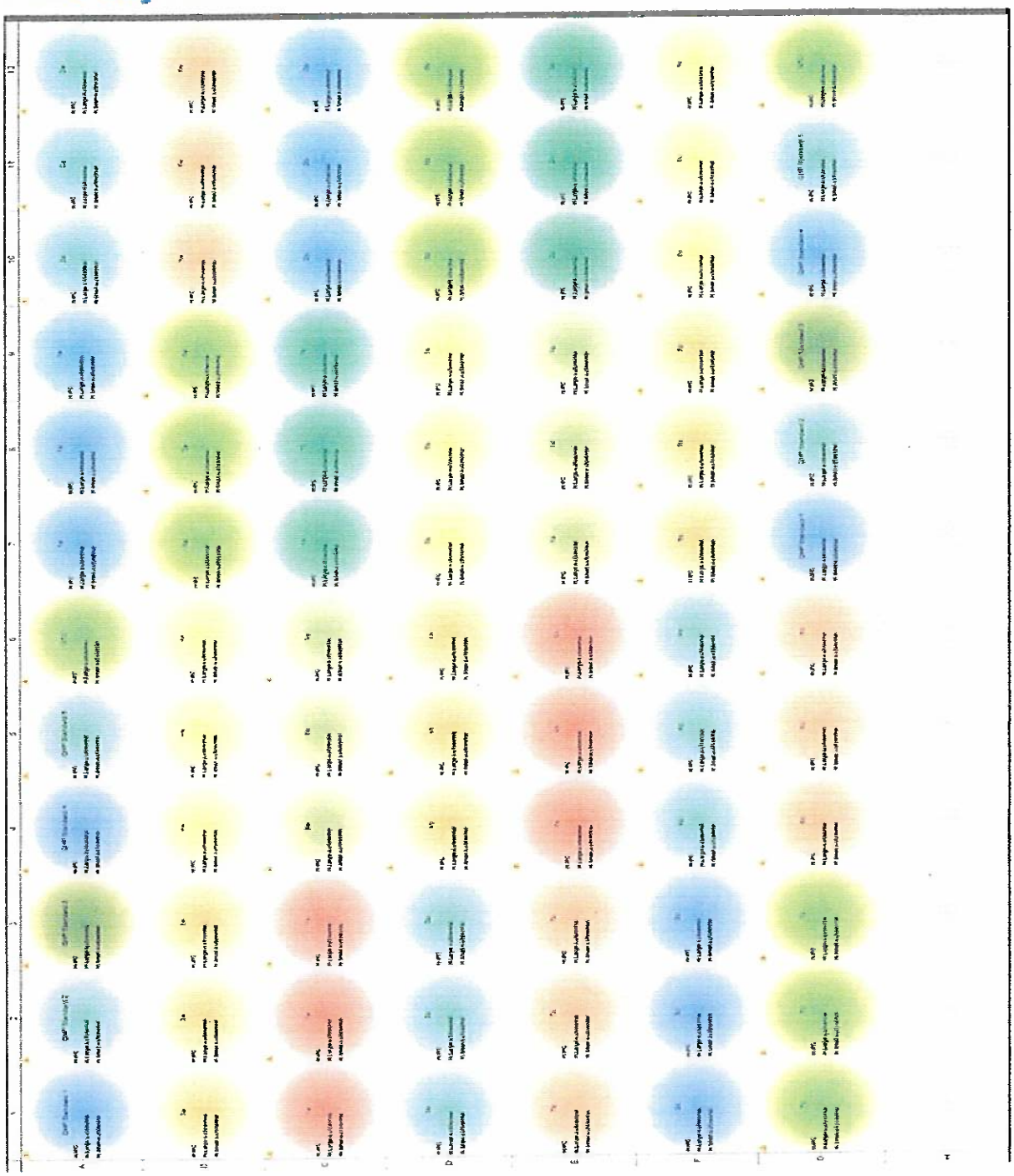
Experiment Results Report

20437913GA

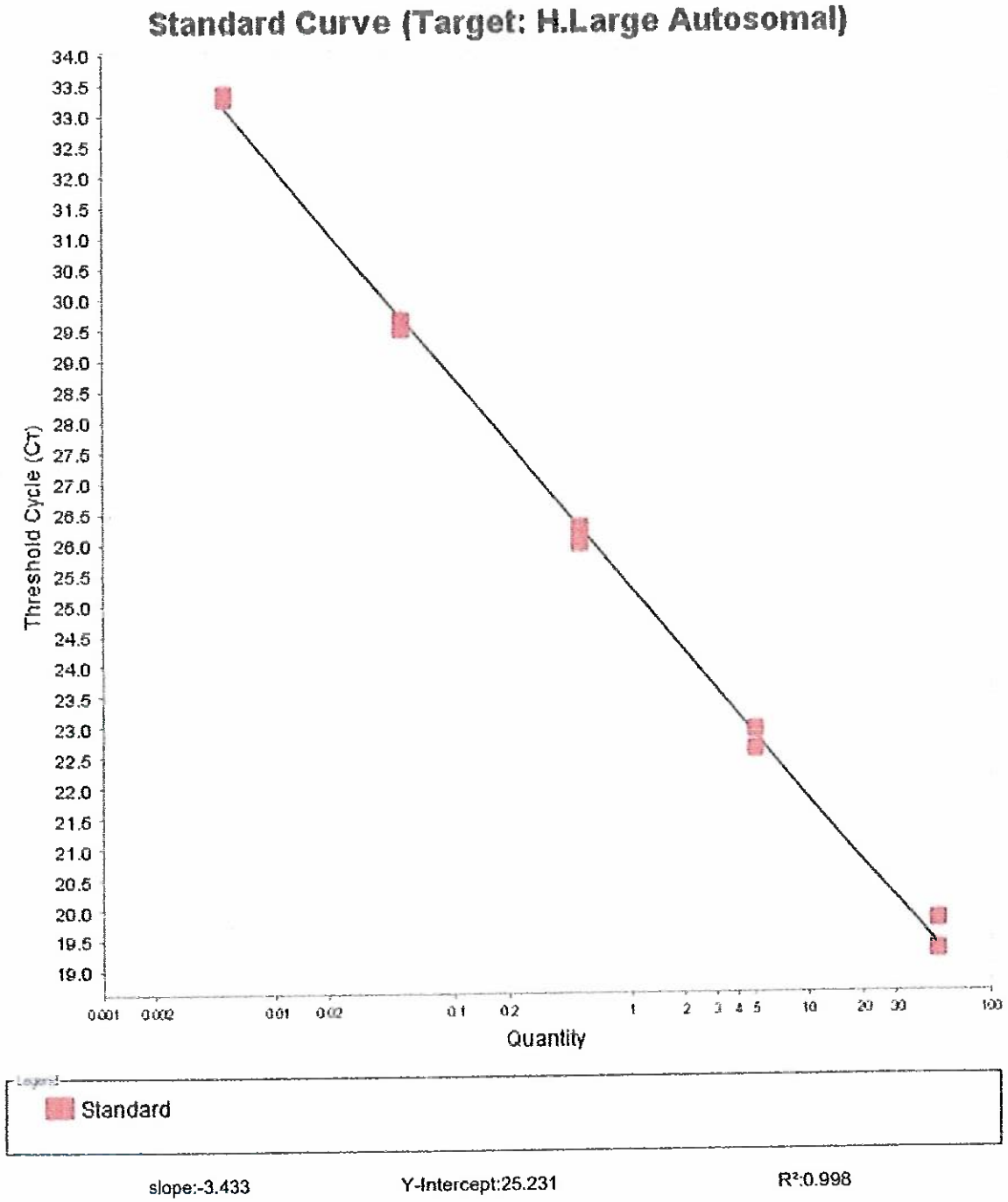
Experiment Summary

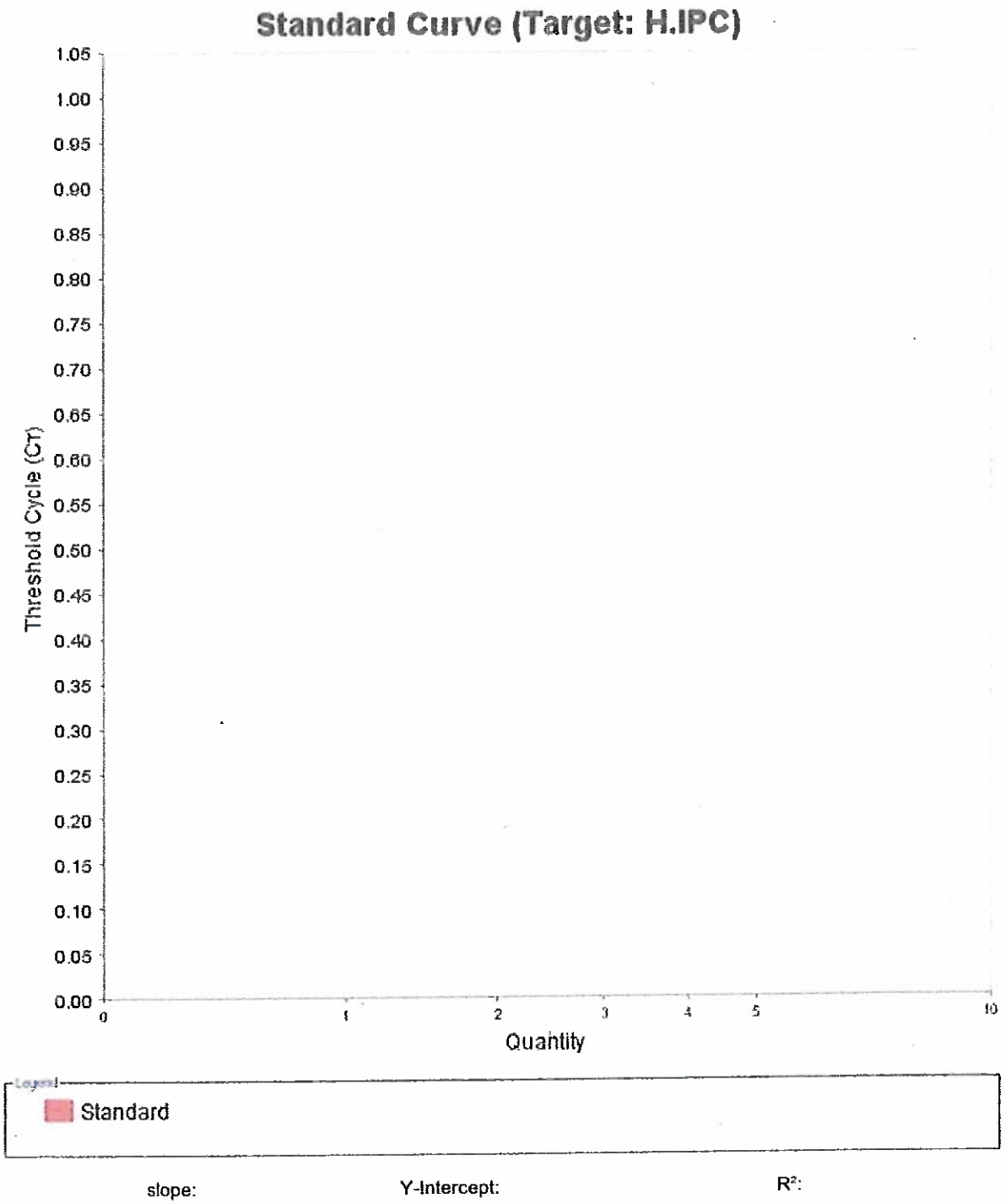
Experiment Name	:20437913GA
Experiment Type	:Quantitation - HID Standard Curve
Kit Name	:Quantifiler Human Plus
File Name	:sml03272024.eds
Run Started	:2024 Mar 27 12:35:05 PM
Run Finished	:2024 Mar 27 1:25:11 PM
Run Duration	:50 minutes 6 seconds
Date Modified	:
User	:
Number of wells used	:84
Number of wells with results	:84
Instrument Name	:2725212083
Instrument Type	:QuantStudio™ 5 System
Comments	:

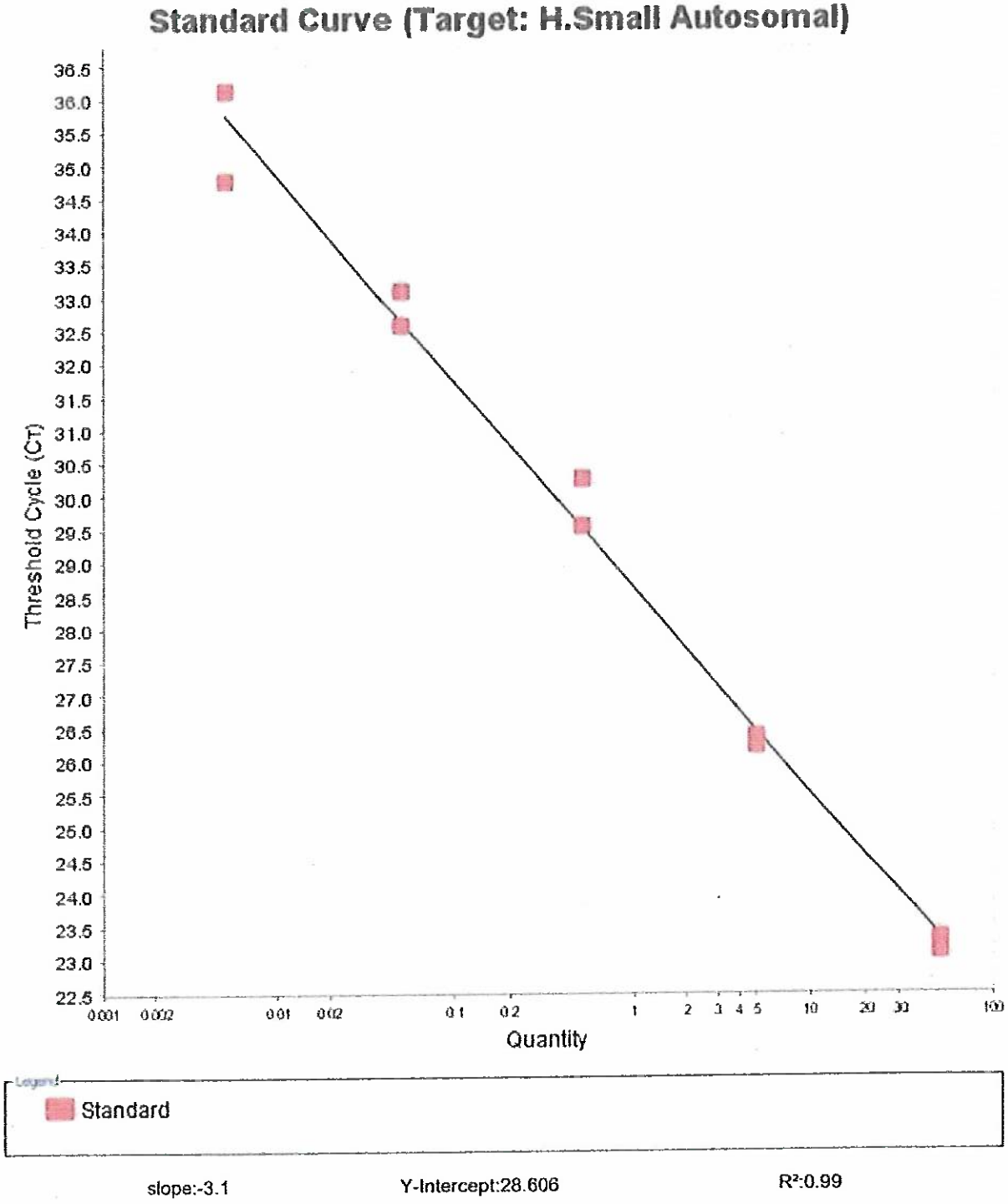
Plate Layout



Standard Curve

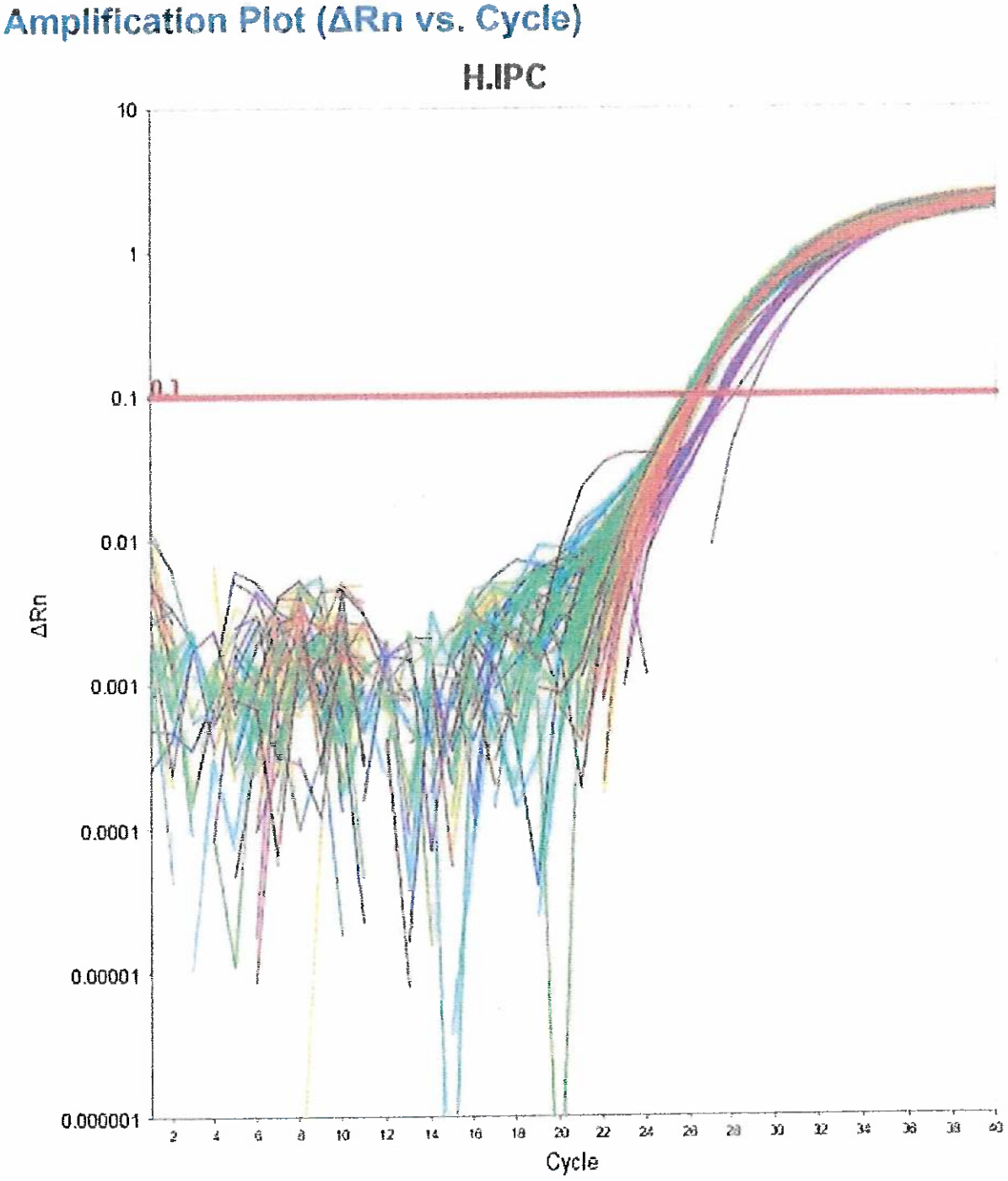



Standard Curve (Target: H.IPC)
Threshold Cycle (Ct)
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
10
Quantity
Standard
slope:
Y-Intercept:
R²:

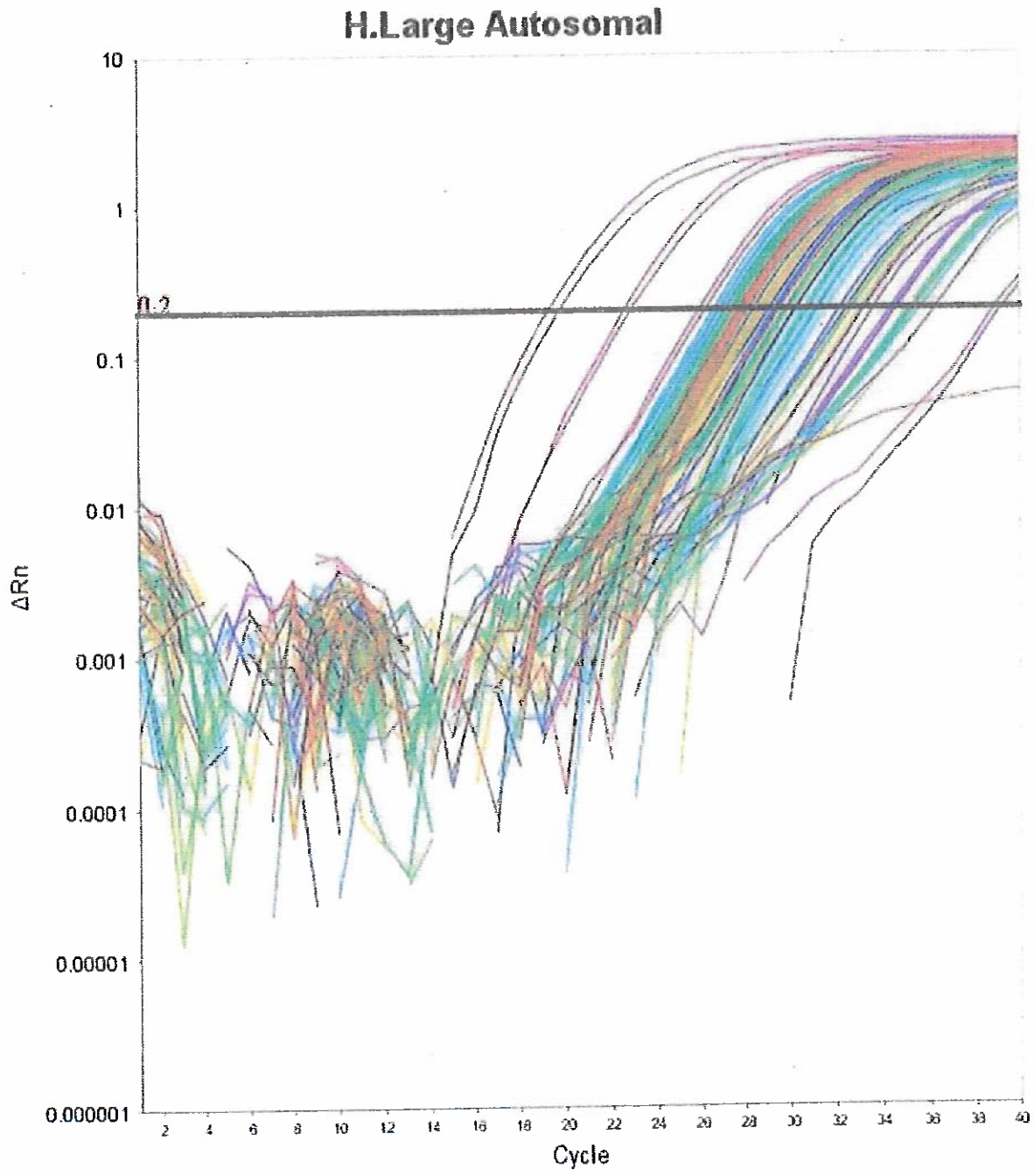

Standard Curve (Target: H.Small Autosomal)
Threshold Cycle (CT)
36.5
36.0
35.5
35.0
34.5
34.0
33.5
33.0
32.5
32.0
31.5
31.0
30.5
30.0
29.5
29.0
28.5
28.0
27.5
27.0
26.5
26.0
25.5
25.0
24.5
24.0
23.5
23.0
22.5
0.001
0.002
0.01
0.02
0.1
0.2
1
2
3
4
5
10
20
30
100
Quantity
Standard
slope:-3.1
Y-Intercept:28.606
R²:0.99

Virtual Standard Curve Summary

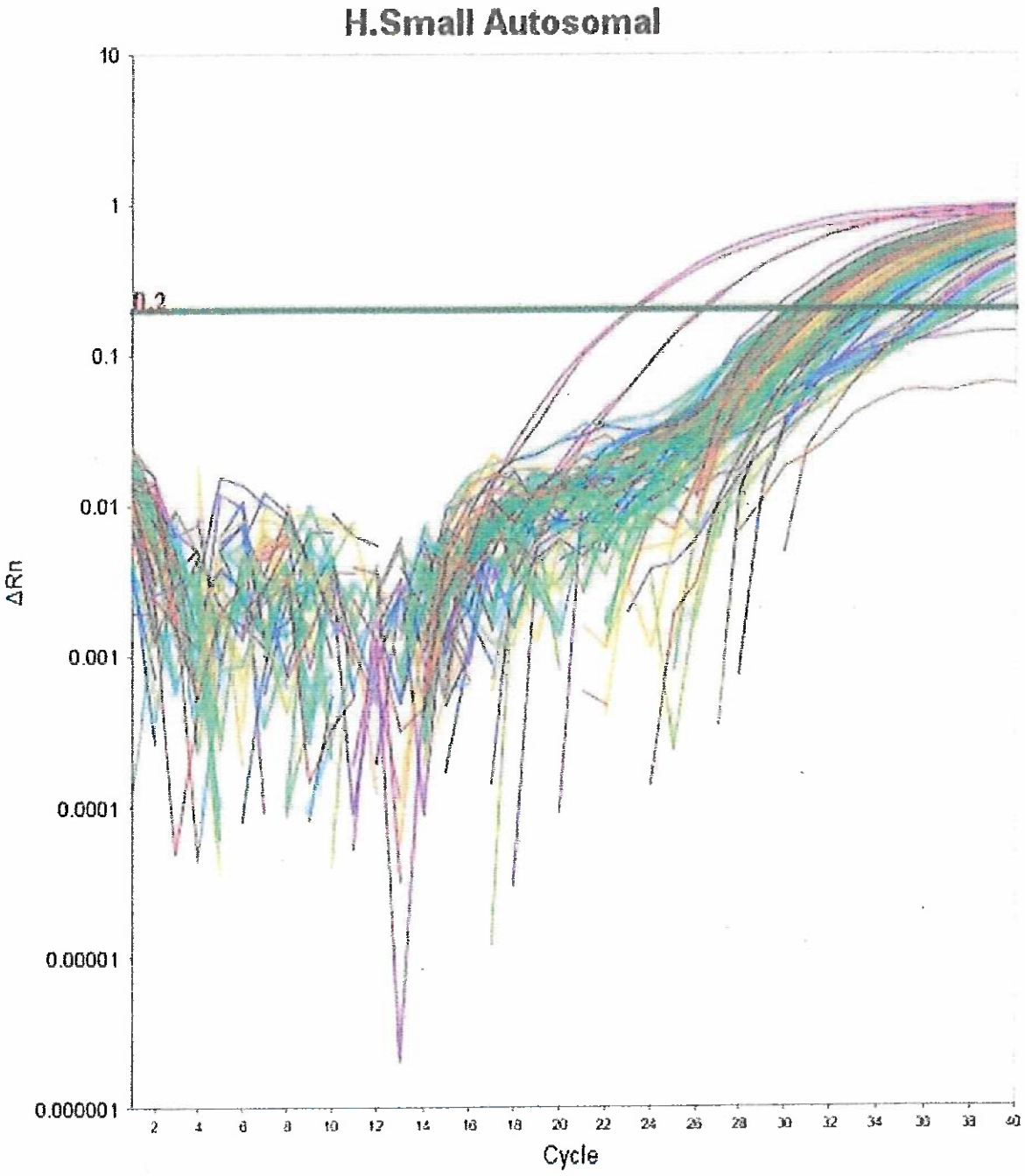
Virtual Standard Curve Name	:
Expiration Date	:
Kit Name	:
Target Details	:


Amplification Plot (ΔRn vs. Cycle)
H.IPC
10
1
0.1
0.1
0.01
0.001
0.0001
0.00001
0.000001
ΔRn
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle

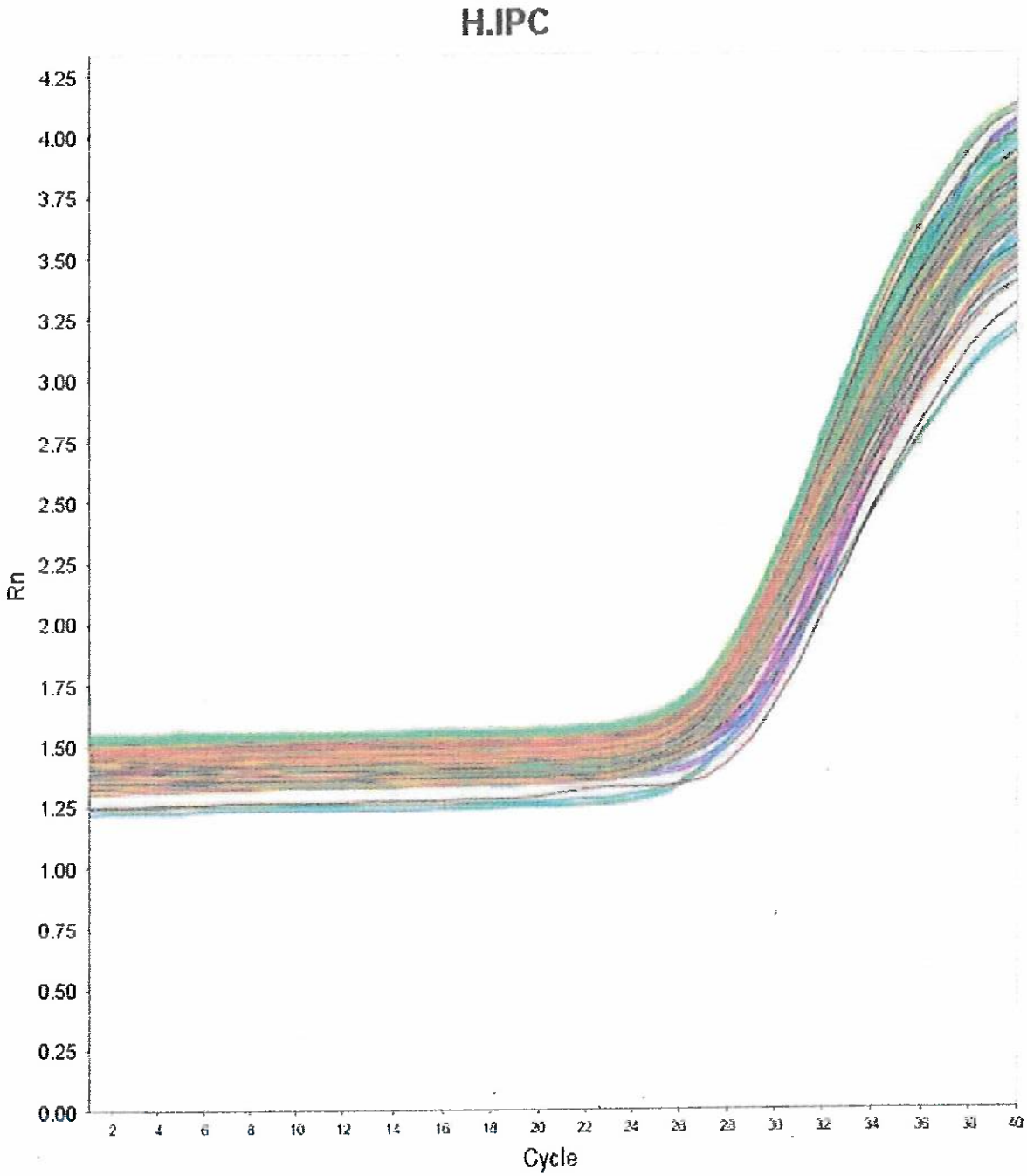
Amplification Plot (ΔRn vs. Cycle)



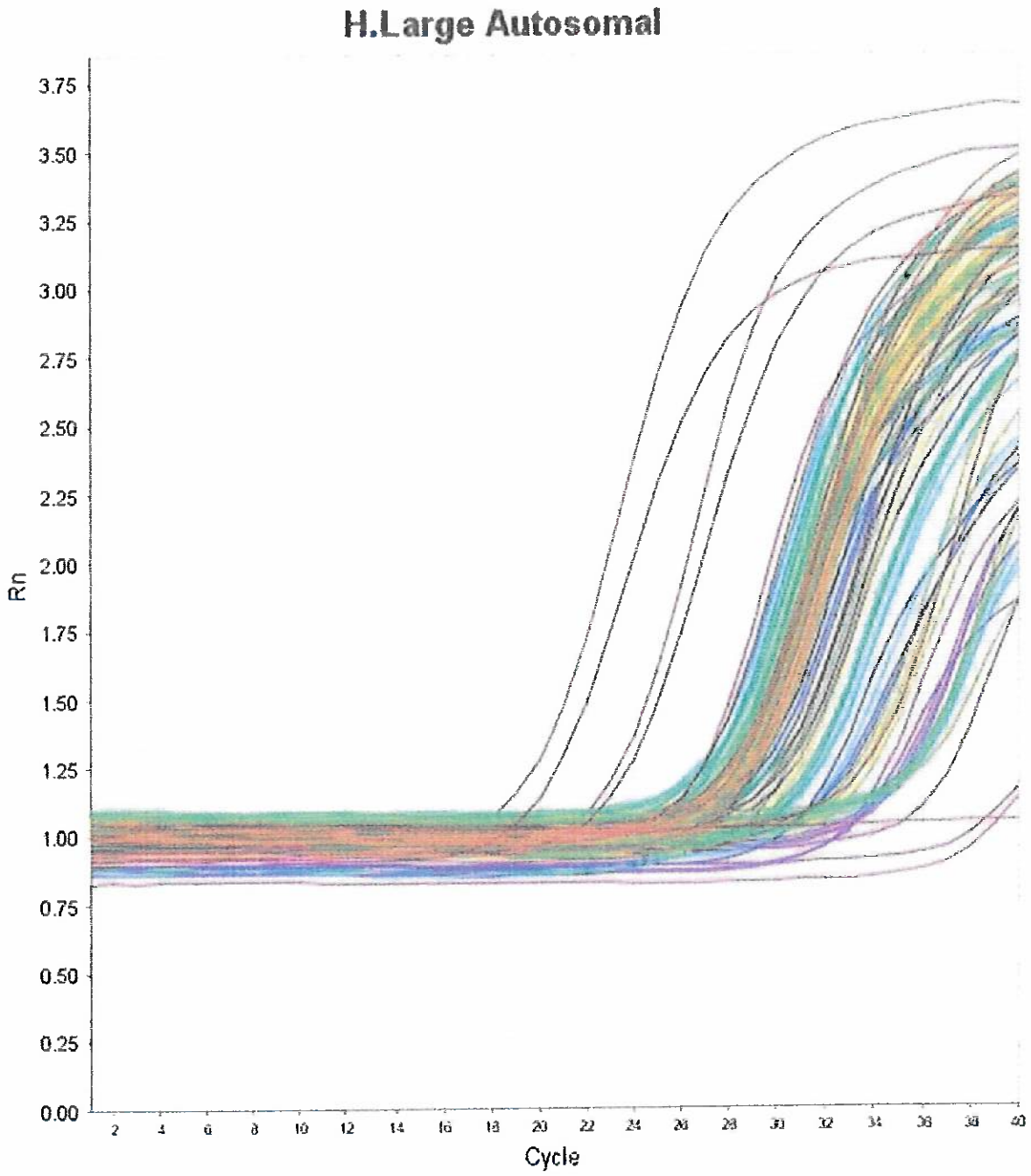
Amplification Plot (ΔRn vs. Cycle)



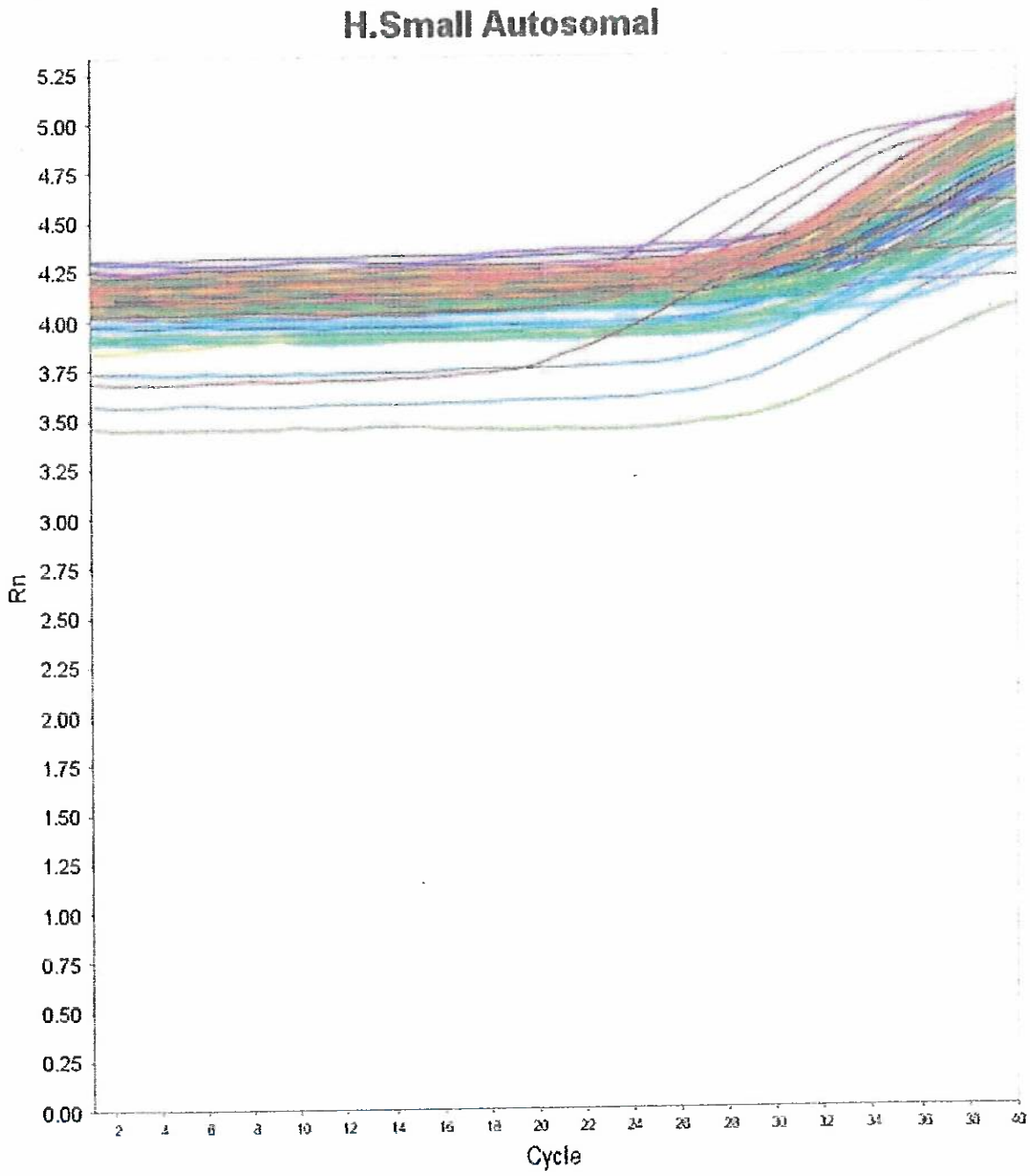
Amplification Plot (Rn vs. Cycle)



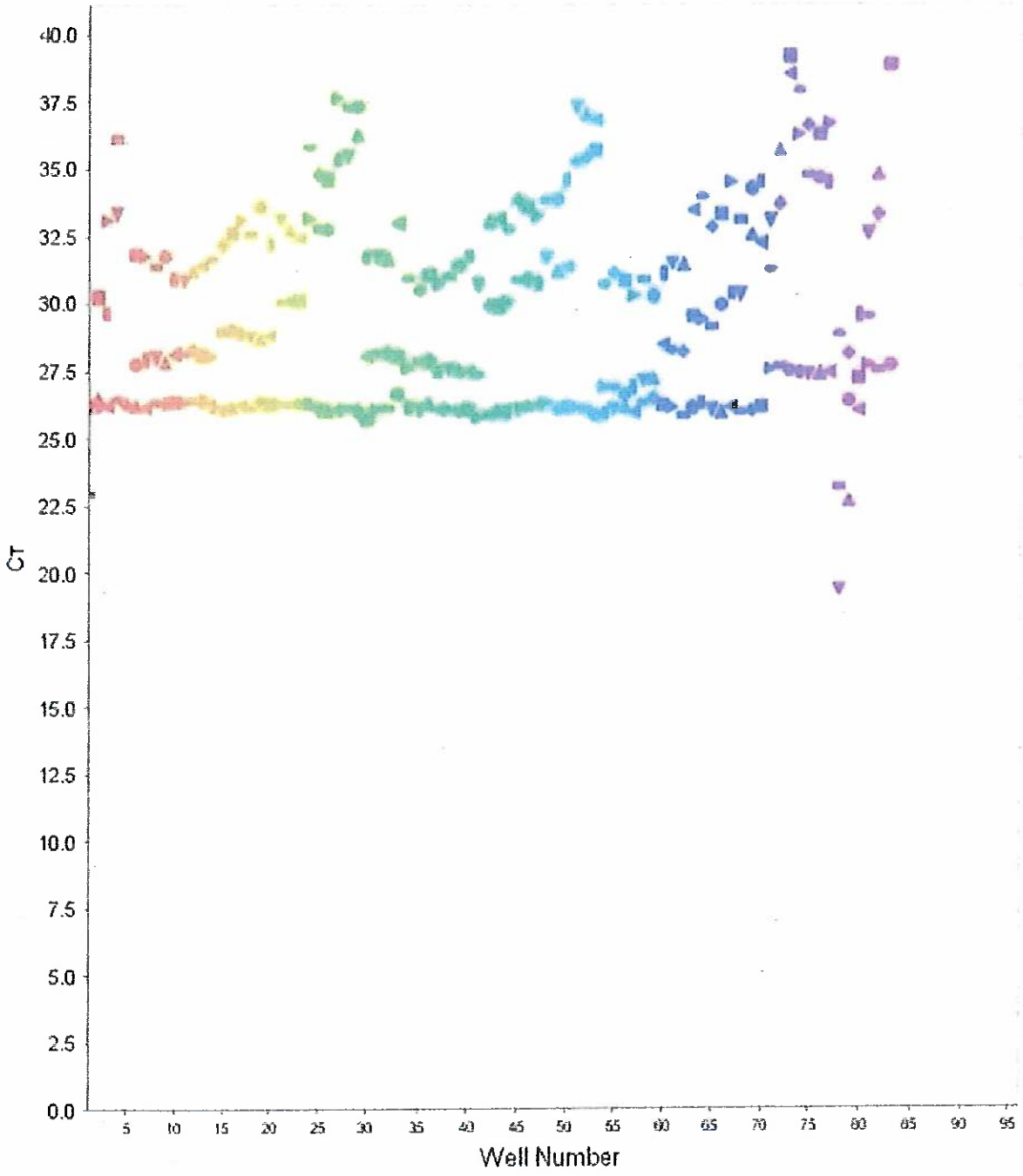
Amplification Plot (Rn vs. Cycle)



Amplification Plot (Rn vs. Cycle)



Amplification Plot (C_T vs. Well)



Results Table

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
A1	QHP Standard 1	H.IPC	Unk				27.5799	28.21	0.89
A1	QHP Standard 1	H.Large Autosomal	Std	50			19.7879	19.55	0.34
A1	QHP Standard 1	H.Small Autosomal	Std	50			23.3111	23.22	0.13
A2	QHP Standard 2	H.IPC	Unk				26.0941	27.09	1.41
A2	QHP Standard 2	H.Large Autosomal	Std	5			22.9309	22.77	0.22
A2	QHP Standard 2	H.Small Autosomal	Std	5			26.225	26.29	0.10
A3	QHP Standard 3	H.IPC	Unk				26.4392	26.81	0.52
A3	QHP Standard 3	H.Large Autosomal	Std	0.5			26.2418	26.11	0.18
A3	QHP Standard 3	H.Small Autosomal	Std	0.5			30.2502	29.90	0.49
A4	QHP Standard 4	H.IPC	Unk				26.2197	26.96	1.05
A4	QHP Standard 4	H.Large Autosomal	Std	0.05			29.6414	29.57	0.10
A4	QHP Standard 4	H.Small Autosomal	Std	0.05			33.1058	32.84	0.38
A5	QHP Standard 5	H.IPC	Unk				26.4226	26.97	0.78
A5	QHP Standard 5	H.Large Autosomal	Std	0.005			33.3548	33.32	0.05
A5	QHP Standard 5	H.Small Autosomal	Std	0.005			36.126	35.45	0.96
A6	NTC	H.IPC	Unk				26.2515	26.95	0.99
A6	NTC	H.Large Autosomal	NTC				Undetermined		
A6	NTC	H.Small Autosomal	NTC				Undetermined		
A7	1a	H.IPC	Unk				26.1999	26.22	0.10
A7	1a	H.Large Autosomal	Unk	0.1831	0.17	0.01	27.7618	27.89	0.12
A7	1a	H.Small Autosomal	Unk	0.0898	0.10	0.02	31.8513	31.66	0.26
A8	1a	H.IPC	Unk				26.1404	26.22	0.10
A8	1a	H.Large Autosomal	Unk	0.1638	0.17	0.01	27.9275	27.89	0.12
A8	1a	H.Small Autosomal	Unk	0.0955	0.10	0.02	31.7681	31.66	0.26
A9	1a	H.IPC	Unk				26.3314	26.22	0.10

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
A9	1a	H.Large Autosomal	Unk	0.1569	0.17	0.01	27.9919	27.89	0.12
A9	1a	H.Small Autosomal	Unk	0.1294	0.10	0.02	31.3597	31.66	0.26
A10	2a	H.IPC	Unk				26.2874	26.32	0.03
A10	2a	H.Large Autosomal	Unk	0.1747	0.15	0.02	27.8314	28.03	0.17
A10	2a	H.Small Autosomal	Unk	0.095	0.15	0.05	31.7756	31.18	0.52
A11	2a	H.IPC	Unk				26.3282	26.32	0.03
A11	2a	H.Large Autosomal	Unk	0.1431	0.15	0.02	28.129	28.03	0.17
A11	2a	H.Small Autosomal	Unk	0.1808	0.15	0.05	30.9091	31.18	0.52
A12	2a	H.IPC	Unk				26.3498	26.32	0.03
A12	2a	H.Large Autosomal	Unk	0.1444	0.15	0.02	28.1153	28.03	0.17
A12	2a	H.Small Autosomal	Unk	0.1886	0.15	0.05	30.8527	31.18	0.52
B1	3a	H.IPC	Unk				26.348	26.29	0.11
B1	3a	H.Large Autosomal	Unk	0.1287	0.14	0.01	28.2873	28.12	0.15
B1	3a	H.Small Autosomal	Unk	0.144	0.12	0.02	31.2158	31.43	0.22
B2	3a	H.IPC	Unk				26.373	26.29	0.11
B2	3a	H.Large Autosomal	Unk	0.1503	0.14	0.01	28.0554	28.12	0.15
B2	3a	H.Small Autosomal	Unk	0.1226	0.12	0.02	31.4319	31.43	0.22
B3	3a	H.IPC	Unk				26.164	26.29	0.11
B3	3a	H.Large Autosomal	Unk	0.1546	0.14	0.01	28.014	28.12	0.15
B3	3a	H.Small Autosomal	Unk	0.104	0.12	0.02	31.6541	31.43	0.22
B4	4a	H.IPC	Unk				25.9913	26.11	0.11
B4	4a	H.Large Autosomal	Unk	0.0826	0.08	0.00	28.9476	28.94	0.06
B4	4a	H.Small Autosomal	Unk	0.0717	0.05	0.02	32.1553	32.61	0.46
B5	4a	H.IPC	Unk				26.1452	26.11	0.11
B5	4a	H.Large Autosomal	Unk	0.0801	0.08	0.00	28.9947	28.94	0.06
B5	4a	H.Small Autosomal	Unk	0.0511	0.05	0.02	32.6115	32.61	0.46

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
B6	4a	H.IPC	Unk				26.1973	26.11	0.11
B6	4a	H.Large Autosomal	Unk	0.087	0.08	0.00	28.871	28.94	0.06
B6	4a	H.Small Autosomal	Unk	0.0361	0.05	0.02	33.0774	32.61	0.46
B7	5a	H.IPC	Unk				26.0755	26.22	0.13
B7	5a	H.Large Autosomal	Unk	0.0946	0.09	0.00	28.7457	28.75	0.06
B7	5a	H.Small Autosomal	Unk	0.0524	0.05	0.02	32.5775	32.77	0.72
B8	5a	H.IPC	Unk				26.3393	26.22	0.13
B8	5a	H.Large Autosomal	Unk	0.0984	0.09	0.00	28.6874	28.75	0.06
B8	5a	H.Small Autosomal	Unk	0.0251	0.05	0.02	33.5677	32.77	0.72
B9	5a	H.IPC	Unk				26.2401	26.22	0.13
B9	5a	H.Large Autosomal	Unk	0.091	0.09	0.00	28.8046	28.75	0.06
B9	5a	H.Small Autosomal	Unk	0.0704	0.05	0.02	32.1786	32.77	0.72
B10	6a	H.IPC	Unk				26.252	26.26	0.01
B10	6a	H.Large Autosomal	Unk	0.0402	0.04	0.00	30.0219	30.07	0.04
B10	6a	H.Small Autosomal	Unk	0.0355	0.05	0.01	33.1013	32.75	0.34
B11	6a	H.IPC	Unk				26.255	26.26	0.01
B11	6a	H.Large Autosomal	Unk	0.0387	0.04	0.00	30.0798	30.07	0.04
B11	6a	H.Small Autosomal	Unk	0.0463	0.05	0.01	32.7428	32.75	0.34
B12	6a	H.IPC	Unk				26.2702	26.26	0.01
B12	6a	H.Large Autosomal	Unk	0.0379	0.04	0.00	30.109	30.07	0.04
B12	6a	H.Small Autosomal	Unk	0.0592	0.05	0.01	32.4136	32.75	0.34
C1	7a	H.IPC	Unk				26.2649	26.11	0.14
C1	7a	H.Large Autosomal	Unk	0.0049	0.01	0.00	33.1528	32.90	0.22
C1	7a	H.Small Autosomal	Unk	0.0047	0.01	0.00	35.8299	35.07	0.66
C2	7a	H.IPC	Unk				26.0238	26.11	0.14
C2	7a	H.Large Autosomal	Unk	0.0062	0.01	0.00	32.811	32.90	0.22

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
C2	7a	H.Small Autosomal	Unk	0.0103	0.01	0.00	34.7733	35.07	0.66
C3	7a	H.IPC	Unk				26.0298	26.11	0.14
C3	7a	H.Large Autosomal	Unk	0.0065	0.01	0.00	32.7331	32.90	0.22
C3	7a	H.Small Autosomal	Unk	0.0116	0.01	0.00	34.6079	35.07	0.66
C4	8a	H.IPC	Unk				26.0297	26.06	0.05
C4	8a	H.Large Autosomal	Unk	0.0011	0.00	0.00	35.3397	35.68	0.50
C4	8a	H.Small Autosomal	Unk	0.0012	0.00	0.00	37.6438	37.41	0.20
C5	8a	H.IPC	Unk				26.1152	26.06	0.05
C5	8a	H.Large Autosomal	Unk	0.0011	0.00	0.00	35.4499	35.68	0.50
C5	8a	H.Small Autosomal	Unk	0.0016	0.00	0.00	37.2761	37.41	0.20
C6	8a	H.IPC	Unk				26.0294	26.06	0.05
C6	8a	H.Large Autosomal	Unk	0.0006	0.00	0.00	36.2574	35.68	0.50
C6	8a	H.Small Autosomal	Unk	0.0016	0.00	0.00	37.3107	37.41	0.20
C7	1b	H.IPC	Unk				25.6985	25.95	0.22
C7	1b	H.Large Autosomal	Unk	0.1514	0.15	0.00	28.0447	28.10	0.05
C7	1b	H.Small Autosomal	Unk	0.1008	0.10	0.00	31.6952	31.70	0.04
C8	1b	H.IPC	Unk				26.0671	25.95	0.22
C8	1b	H.Large Autosomal	Unk	0.1452	0.15	0.00	28.107	28.10	0.05
C8	1b	H.Small Autosomal	Unk	0.0979	0.10	0.00	31.7357	31.70	0.04
C9	1b	H.IPC	Unk				26.0761	25.95	0.22
C9	1b	H.Large Autosomal	Unk	0.1422	0.15	0.00	28.1388	28.10	0.05
C9	1b	H.Small Autosomal	Unk	0.1036	0.10	0.00	31.6588	31.70	0.04
C10	2b	H.IPC	Unk				26.6448	26.27	0.33
C10	2b	H.Large Autosomal	Unk	0.1523	0.18	0.03	28.0361	27.83	0.22
C10	2b	H.Small Autosomal	Unk	0.0388	0.15	0.11	32.982	31.47	1.33
C11	2b	H.IPC	Unk				26.1254	26.27	0.33

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	Ct	Ct (Mean)	Ct (Std Dev)
C11	2b	H.Large Autosomal	Unk	0.2038	0.18	0.03	27.6022	27.83	0.22
C11	2b	H.Small Autosomal	Unk	0.1779	0.15	0.11	30.9307	31.47	1.33
C12	2b	H.IPC	Unk				26.0316	26.27	0.33
C12	2b	H.Large Autosomal	Unk	0.1726	0.18	0.03	27.8497	27.83	0.22
C12	2b	H.Small Autosomal	Unk	0.2452	0.15	0.11	30.4988	31.47	1.33
D1	3b	H.IPC	Unk				26.2616	26.14	0.10
D1	3b	H.Large Autosomal	Unk	0.1719	0.20	0.03	27.8559	27.62	0.21
D1	3b	H.Small Autosomal	Unk	0.1637	0.18	0.02	31.0432	30.95	0.17
D2	3b	H.IPC	Unk				26.0766	26.14	0.10
D2	3b	H.Large Autosomal	Unk	0.2261	0.20	0.03	27.4473	27.62	0.21
D2	3b	H.Small Autosomal	Unk	0.2037	0.18	0.02	30.7485	30.95	0.17
D3	3b	H.IPC	Unk				26.0855	26.14	0.10
D3	3b	H.Large Autosomal	Unk	0.2122	0.20	0.03	27.5418	27.62	0.21
D3	3b	H.Small Autosomal	Unk	0.1631	0.18	0.02	31.0483	30.95	0.17
D4	4b	H.IPC	Unk				25.9937	25.95	0.14
D4	4b	H.Large Autosomal	Unk	0.2104	0.22	0.01	27.5545	27.46	0.09
D4	4b	H.Small Autosomal	Unk	0.1237	0.15	0.06	31.4205	31.28	0.55
D5	4b	H.IPC	Unk				26.0568	25.95	0.14
D5	4b	H.Large Autosomal	Unk	0.2237	0.22	0.01	27.4633	27.46	0.09
D5	4b	H.Small Autosomal	Unk	0.0972	0.15	0.06	31.7454	31.28	0.55
D6	4b	H.IPC	Unk				25.7896	25.95	0.14
D6	4b	H.Large Autosomal	Unk	0.2387	0.22	0.01	27.3663	27.46	0.09
D6	4b	H.Small Autosomal	Unk	0.2154	0.15	0.06	30.6737	31.28	0.55
D7	5b	H.IPC	Unk				25.8511	25.94	0.08
D7	5b	H.Large Autosomal	Unk	0.0432	0.04	0.00	29.9162	29.95	0.08
D7	5b	H.Small Autosomal	Unk	0.0363	0.04	0.01	33.0723	32.99	0.21

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
D8	5b	H.IPC	Unk				26.0062	25.94	0.08
D8	5b	H.Large Autosomal	Unk	0.0435	0.04	0.00	29.9035	29.95	0.08
D8	5b	H.Small Autosomal	Unk	0.0344	0.04	0.01	33.1428	32.99	0.21
D9	5b	H.IPC	Unk				25.9548	25.94	0.08
D9	5b	H.Large Autosomal	Unk	0.0397	0.04	0.00	30.0408	29.95	0.08
D9	5b	H.Small Autosomal	Unk	0.0459	0.04	0.01	32.7554	32.99	0.21
D10	6b	H.IPC	Unk				26.0804	26.16	0.08
D10	6b	H.Large Autosomal	Unk	0.0227	0.02	0.00	30.8746	30.83	0.09
D10	6b	H.Small Autosomal	Unk	0.0207	0.03	0.01	33.8291	33.56	0.28
D11	6b	H.IPC	Unk				26.1868	26.16	0.08
D11	6b	H.Large Autosomal	Unk	0.0225	0.02	0.00	30.8876	30.83	0.09
D11	6b	H.Small Autosomal	Unk	0.0249	0.03	0.01	33.5808	33.56	0.28
D12	6b	H.IPC	Unk				26.2268	26.16	0.08
D12	6b	H.Large Autosomal	Unk	0.025	0.02	0.00	30.7301	30.83	0.09
D12	6b	H.Small Autosomal	Unk	0.0314	0.03	0.01	33.2674	33.56	0.28
E1	7b	H.IPC	Unk				26.2997	26.16	0.12
E1	7b	H.Large Autosomal	Unk	0.0132	0.02	0.00	31.6843	31.42	0.24
E1	7b	H.Small Autosomal	Unk	0.0206	0.02	0.00	33.8333	34.10	0.41
E2	7b	H.IPC	Unk				26.0573	26.16	0.12
E2	7b	H.Large Autosomal	Unk	0.0181	0.02	0.00	31.209	31.42	0.24
E2	7b	H.Small Autosomal	Unk	0.0198	0.02	0.00	33.8899	34.10	0.41
E3	7b	H.IPC	Unk				26.1256	26.16	0.12
E3	7b	H.Large Autosomal	Unk	0.0164	0.02	0.00	31.3576	31.42	0.24
E3	7b	H.Small Autosomal	Unk	0.012	0.02	0.00	34.5666	34.10	0.41
E4	8b	H.IPC	Unk				26.1139	26.01	0.14
E4	8b	H.Large Autosomal	Unk	0.0012	0.00	0.00	35.275	35.47	0.21

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
E4	8b	H.Small Autosomal	Unk	0.0016	0.00	0.00	37.3128	37.06	0.24
E5	8b	H.IPC	Unk				26.0629	26.01	0.14
E5	8b	H.Large Autosomal	Unk	0.0011	0.00	0.00	35.4275	35.47	0.21
E5	8b	H.Small Autosomal	Unk	0.0019	0.00	0.00	37.0389	37.06	0.24
E6	8b	H.IPC	Unk				25.8465	26.01	0.14
E6	8b	H.Large Autosomal	Unk	0.0009	0.00	0.00	35.6951	35.47	0.21
E6	8b	H.Small Autosomal	Unk	0.0022	0.00	0.00	36.8312	37.06	0.24
E7	1c	H.IPC	Unk				25.9097	26.04	0.12
E7	1c	H.Large Autosomal	Unk	0.3248	0.35	0.04	26.9073	26.80	0.16
E7	1c	H.Small Autosomal	Unk	0.2096	0.19	0.03	30.7102	30.88	0.21
E8	1c	H.IPC	Unk				26.0422	26.04	0.12
E8	1c	H.Large Autosomal	Unk	0.3347	0.35	0.04	26.8623	26.80	0.16
E8	1c	H.Small Autosomal	Unk	0.1557	0.19	0.03	31.1109	30.88	0.21
E9	1c	H.IPC	Unk				26.1549	26.04	0.12
E9	1c	H.Large Autosomal	Unk	0.3942	0.35	0.04	26.6184	26.80	0.16
E9	1c	H.Small Autosomal	Unk	0.1915	0.19	0.03	30.8316	30.88	0.21
E10	2c	H.IPC	Unk				25.9542	26.24	0.25
E10	2c	H.Large Autosomal	Unk	0.3313	0.30	0.03	26.8777	27.05	0.16
E10	2c	H.Small Autosomal	Unk	0.2879	0.25	0.06	30.2829	30.48	0.37
E11	2c	H.IPC	Unk				26.3064	26.24	0.25
E11	2c	H.Large Autosomal	Unk	0.2827	0.30	0.03	27.1138	27.05	0.16
E11	2c	H.Small Autosomal	Unk	0.1818	0.25	0.06	30.9018	30.48	0.37
E12	2c	H.IPC	Unk				26.4497	26.24	0.25
E12	2c	H.Large Autosomal	Unk	0.2719	0.30	0.03	27.1719	27.05	0.16
E12	2c	H.Small Autosomal	Unk	0.2952	0.25	0.06	30.2493	30.48	0.37
F1	3c	H.IPC	Unk				26.1851	26.04	0.18

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
F1	3c	H.Large Autosomal	Unk	0.115	0.13	0.01	28.4554	28.30	0.14
F1	3c	H.Small Autosomal	Unk	0.1591	0.13	0.02	31.0816	31.33	0.22
F2	3c	H.IPC	Unk				26.1072	26.04	0.18
F2	3c	H.Large Autosomal	Unk	0.1325	0.13	0.01	28.2434	28.30	0.14
F2	3c	H.Small Autosomal	Unk	0.1186	0.13	0.02	31.4775	31.33	0.22
F3	3c	H.IPC	Unk				25.8415	26.04	0.18
F3	3c	H.Large Autosomal	Unk	0.1377	0.13	0.01	28.1865	28.30	0.14
F3	3c	H.Small Autosomal	Unk	0.1221	0.13	0.02	31.438	31.33	0.22
F4	4c	H.IPC	Unk				26.1001	26.13	0.13
F4	4c	H.Large Autosomal	Unk	0.0562	0.06	0.01	29.5227	29.36	0.19
F4	4c	H.Small Autosomal	Unk	0.0278	0.03	0.01	33.4296	33.40	0.57
F5	4c	H.IPC	Unk				26.2787	26.13	0.13
F5	4c	H.Large Autosomal	Unk	0.0611	0.06	0.01	29.3971	29.36	0.19
F5	4c	H.Small Autosomal	Unk	0.0188	0.03	0.01	33.9569	33.40	0.57
F6	4c	H.IPC	Unk				26.0241	26.13	0.13
F6	4c	H.Large Autosomal	Unk	0.0722	0.06	0.01	29.1499	29.36	0.19
F6	4c	H.Small Autosomal	Unk	0.0441	0.03	0.01	32.8085	33.40	0.57
F7	5c	H.IPC	Unk				25.9527	26.01	0.11
F7	5c	H.Large Autosomal	Unk	0.0421	0.04	0.01	29.953	30.21	0.23
F7	5c	H.Small Autosomal	Unk	0.0309	0.03	0.01	33.2872	33.62	0.76
F8	5c	H.IPC	Unk				26.1273	26.01	0.11
F8	5c	H.Large Autosomal	Unk	0.0321	0.04	0.01	30.3583	30.21	0.23
F8	5c	H.Small Autosomal	Unk	0.0126	0.03	0.01	34.4906	33.62	0.76
F9	5c	H.IPC	Unk				25.9379	26.01	0.11
F9	5c	H.Large Autosomal	Unk	0.0327	0.04	0.01	30.329	30.21	0.23
F9	5c	H.Small Autosomal	Unk	0.0356	0.03	0.01	33.0958	33.62	0.76

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
F10	6c	H.IPC	Unk				26.0014	26.56	0.85
F10	6c	H.Large Autosomal	Unk	0.0074	0.01	0.01	32.5398	32.00	0.68
F10	6c	H.Small Autosomal	Unk	0.0151	0.02	0.01	34.2491	33.94	0.76
F11	6c	H.IPC	Unk				26.1442	26.56	0.85
F11	6c	H.Large Autosomal	Unk	0.0091	0.01	0.01	32.2297	32.00	0.68
F11	6c	H.Small Autosomal	Unk	0.0126	0.02	0.01	34.5	33.94	0.76
F12	6c	H.IPC	Unk				27.54	26.56	0.85
F12	6c	H.Large Autosomal	Unk	0.0177	0.01	0.01	31.241	32.00	0.68
F12	6c	H.Small Autosomal	Unk	0.0363	0.02	0.01	33.0702	33.94	0.76
G1	7c	H.IPC	Unk				27.639	27.49	0.14
G1	7c	H.Large Autosomal	Unk	0.0034	0.00	0.00	33.693	36.38	2.74
G1	7c	H.Small Autosomal	Unk	0.0051	0.00	0.00	35.7085	37.37	1.47
G2	7c	H.IPC	Unk				27.4557	27.49	0.14
G2	7c	H.Large Autosomal	Unk	0.0001	0.00	0.00	39.1759	36.38	2.74
G2	7c	H.Small Autosomal	Unk	0.0006	0.00	0.00	38.5022	37.37	1.47
G3	7c	H.IPC	Unk				27.3736	27.49	0.14
G3	7c	H.Large Autosomal	Unk	0.0006	0.00	0.00	36.2725	36.38	2.74
G3	7c	H.Small Autosomal	Unk	0.001	0.00	0.00	37.9024	37.37	1.47
G4	8c	H.IPC	Unk				27.3206	27.36	0.04
G4	8c	H.Large Autosomal	Unk	0.0017	0.00	0.00	34.7231	34.61	0.13
G4	8c	H.Small Autosomal	Unk	0.0026	0.00	0.00	36.6367	36.51	0.23
G5	8c	H.IPC	Unk				27.3625	27.36	0.04
G5	8c	H.Large Autosomal	Unk	0.0018	0.00	0.00	34.6448	34.61	0.13
G5	8c	H.Small Autosomal	Unk	0.0034	0.00	0.00	36.2486	36.51	0.23
G6	8c	H.IPC	Unk				27.3933	27.36	0.04
G6	8c	H.Large Autosomal	Unk	0.002	0.00	0.00	34.4748	34.61	0.13

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	C_T	C_T (Mean)	C_T (Std Dev)
G6	8c	H.Small Autosomal	Unk	0.0025	0.00	0.00	36.6559	36.51	0.23
G7	QHP Standard 1	H.IPC	Unk				28.837	28.21	0.89
G7	QHP Standard 1	H.Large Autosomal	Std	50			19.3141	19.55	0.34
G7	QHP Standard 1	H.Small Autosomal	Std	50			23.1237	23.22	0.13
G8	QHP Standard 2	H.IPC	Unk				28.0904	27.09	1.41
G8	QHP Standard 2	H.Large Autosomal	Std	5			22.6136	22.77	0.22
G8	QHP Standard 2	H.Small Autosomal	Std	5			26.364	26.29	0.10
G9	QHP Standard 3	H.IPC	Unk				27.1721	26.81	0.52
G9	QHP Standard 3	H.Large Autosomal	Std	0.5			25.9823	26.11	0.18
G9	QHP Standard 3	H.Small Autosomal	Std	0.5			29.5539	29.90	0.49
G10	QHP Standard 4	H.IPC	Unk				27.7007	26.96	1.05
G10	QHP Standard 4	H.Large Autosomal	Std	0.05			29.4931	29.57	0.10
G10	QHP Standard 4	H.Small Autosomal	Std	0.05			32.5701	32.84	0.38
G11	QHP Standard 5	H.IPC	Unk				27.5235	26.97	0.78
G11	QHP Standard 5	H.Large Autosomal	Std	0.005			33.2799	33.32	0.05
G11	QHP Standard 5	H.Small Autosomal	Std	0.005			34.7675	35.45	0.96
G12	NTC	H.IPC	Unk				27.6551	26.95	0.99
G12	NTC	H.Large Autosomal	NTC				38.8723		
G12	NTC	H.Small Autosomal	NTC				Undetermined		

QC Summary

Total Wells	96	Processed Wells	84	Targets Used	3
Well Setup	84	Flagged Wells	48	Samples Used	30

Flag	Name	Frequency	Locations
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CTFAIL	C_T algorithm failed	0	
DRNMIN	Define acceptable delta Rn based on C_T range	0	
EXPFAIL	Exponential algorithm failed	0	
HIGHQT	High Quantity of DNA	0	
HIGHSD	High standard deviation in replicate group	42	A1, A2, A3, A4, A5, A6, A10, A11, A12, B7, B8, B9, C1, C2, C3, C4, C5, C6, C10, C11, C12, D4, D5, D6, F4, F5, F6, F7, F8, F9, F10, F11, F12, G1, G2, G3, G7, G8, G9, G10, G11, G12
IPCCT	Internal PCR Control C_T value	0	
LOWQT	Low Quantity of DNA	12	C4, C5, C6, E4, E5, E6, G1, G2, G3, G4, G5, G6
NOAMP	No amplification	0	
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
NTCCT	Non-Template Control sample amplification	1	G12
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
PRFDROP	Passive reference signal changes near C_T	0	
PRFLOW	Low passive reference signal	0	
R^2	Low Standard curve R^2 value	0	
SLOPE	Non-optimal slope of the Standard curve	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	
YINT	Y-Intercept	0	

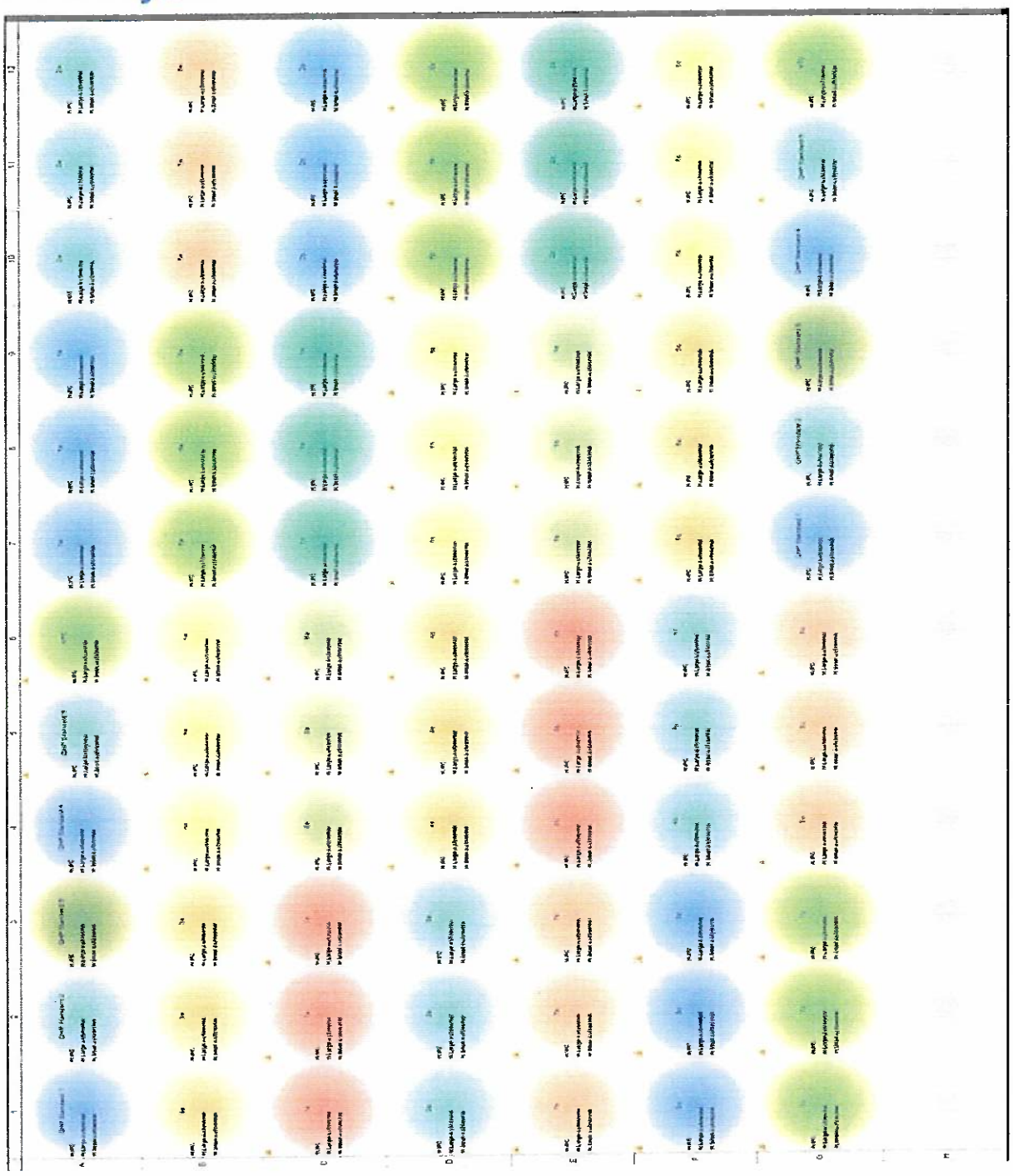
Experiment Results Report

20437913GB

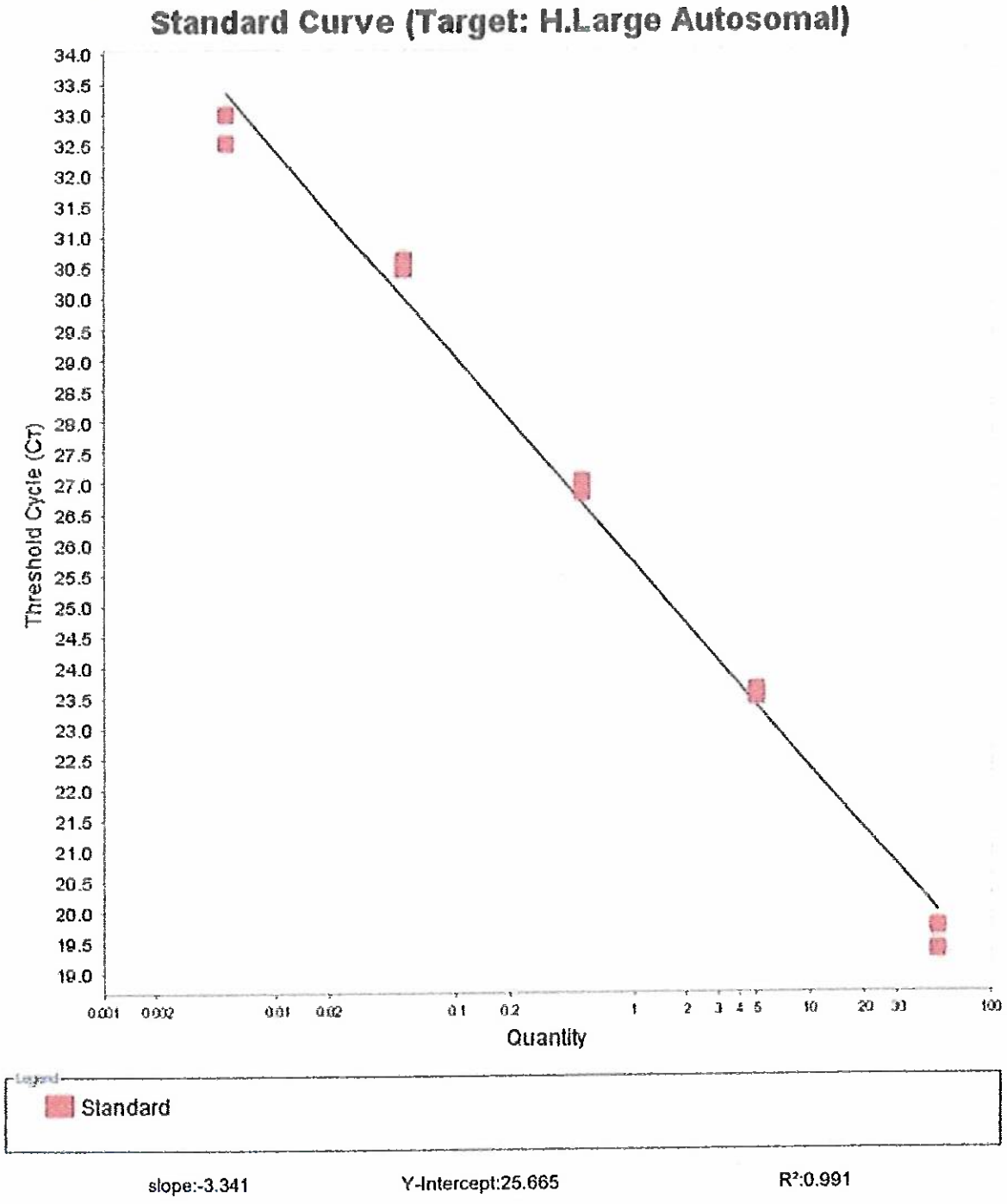
Experiment Summary

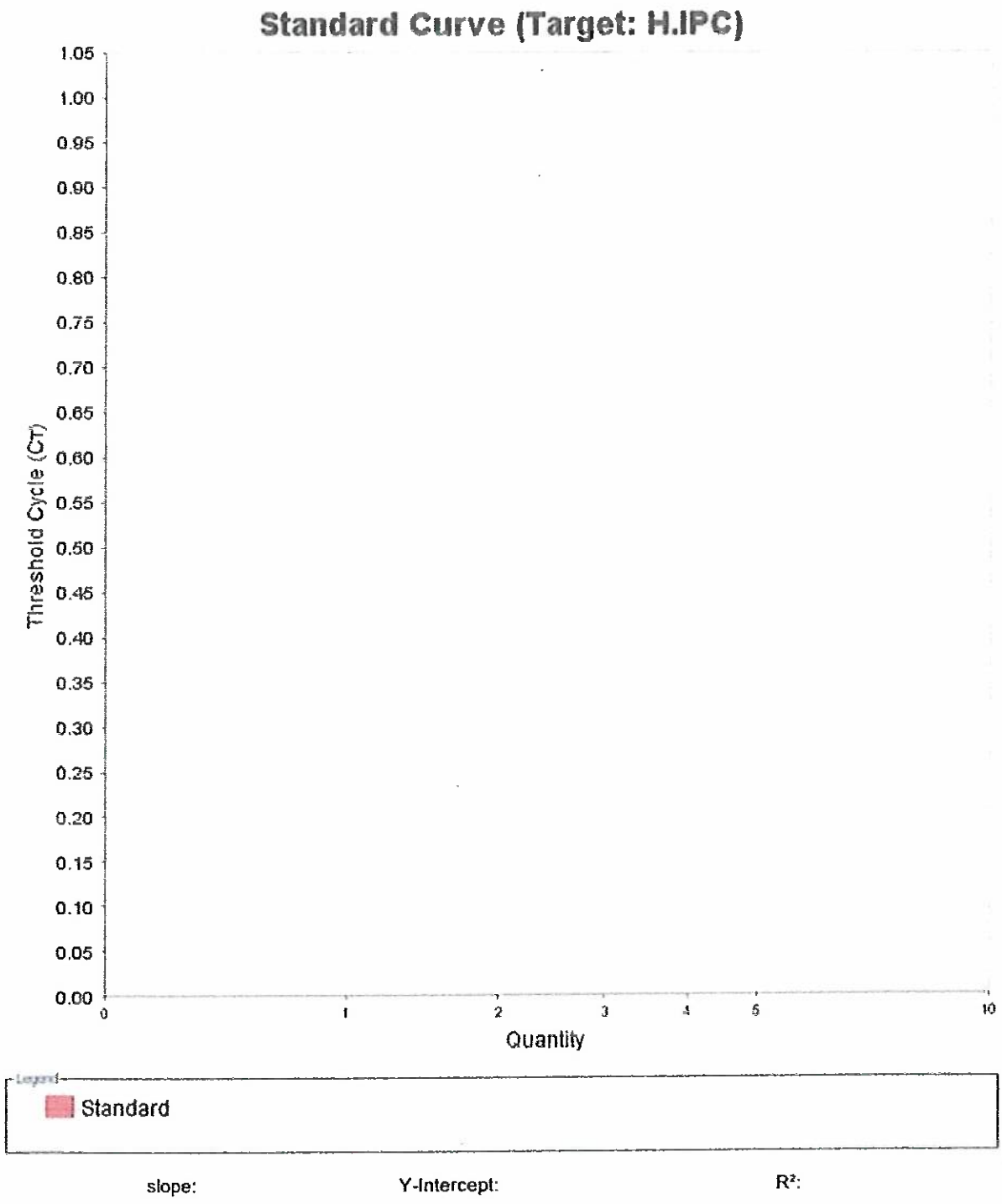
Experiment Name	:20437913GB
Experiment Type	:Quantitation - HID Standard Curve
Kit Name	:Quantifiler Human Plus
File Name	:sml03272024_GB.eds
Run Started	:2024 Mar 27 1:43:24 PM
Run Finished	:2024 Mar 27 2:33:11 PM
Run Duration	:49 minutes 46 seconds
Date Modified	:
User	:
Number of wells used	:84
Number of wells with results	:84
Instrument Name	:2725212083
Instrument Type	:QuantStudio™ 5 System
Comments	:

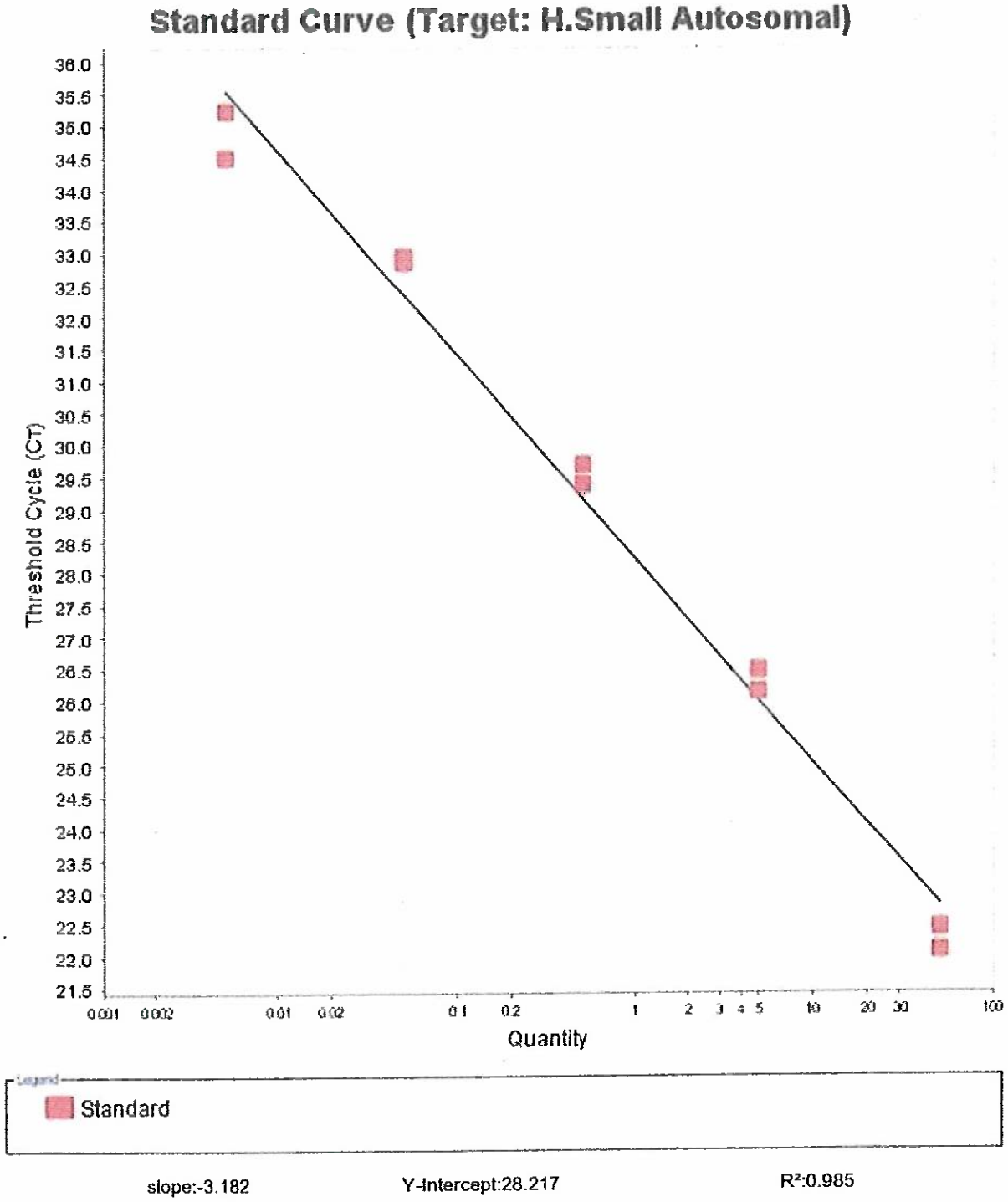
Plate Layout



Standard Curve



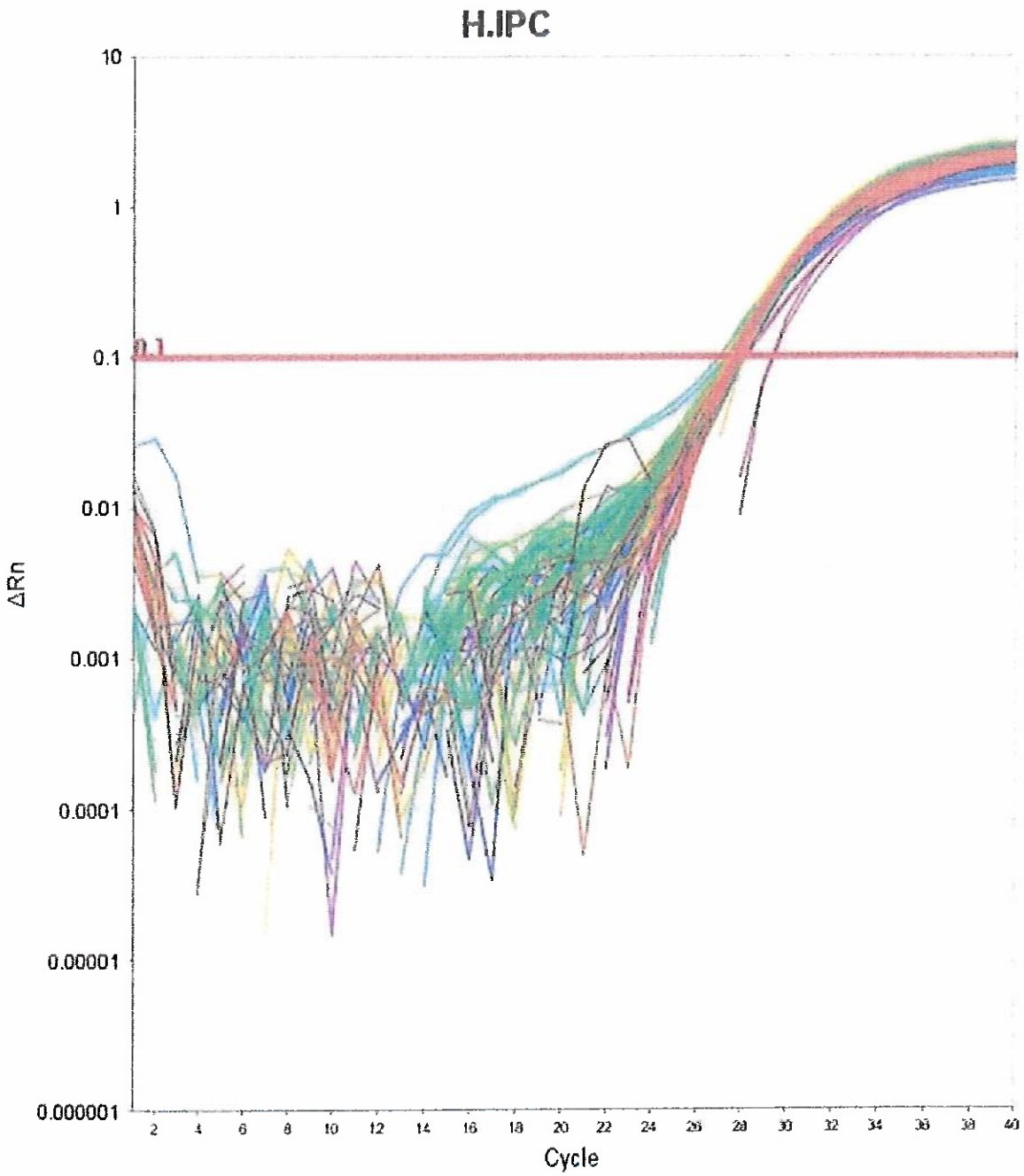

Standard Curve (Target: H.IPC)
Threshold Cycle (Ct)
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
10
Quantity
Legend
Standard
slope:
Y-Intercept:
R²:


Standard Curve (Target: H.Small Autosomal)
Threshold Cycle (CT)
36.0
35.5
35.0
34.5
34.0
33.5
33.0
32.5
32.0
31.5
31.0
30.5
30.0
29.5
29.0
28.5
28.0
27.5
27.0
26.5
26.0
25.5
25.0
24.5
24.0
23.5
23.0
22.5
22.0
21.5
0.001
0.002
0.01
0.02
0.1
0.2
1
2
3
4
5
10
20
30
100
Quantity
Legend
Standard
slope:-3.182
Y-Intercept:28.217
R²:0.985

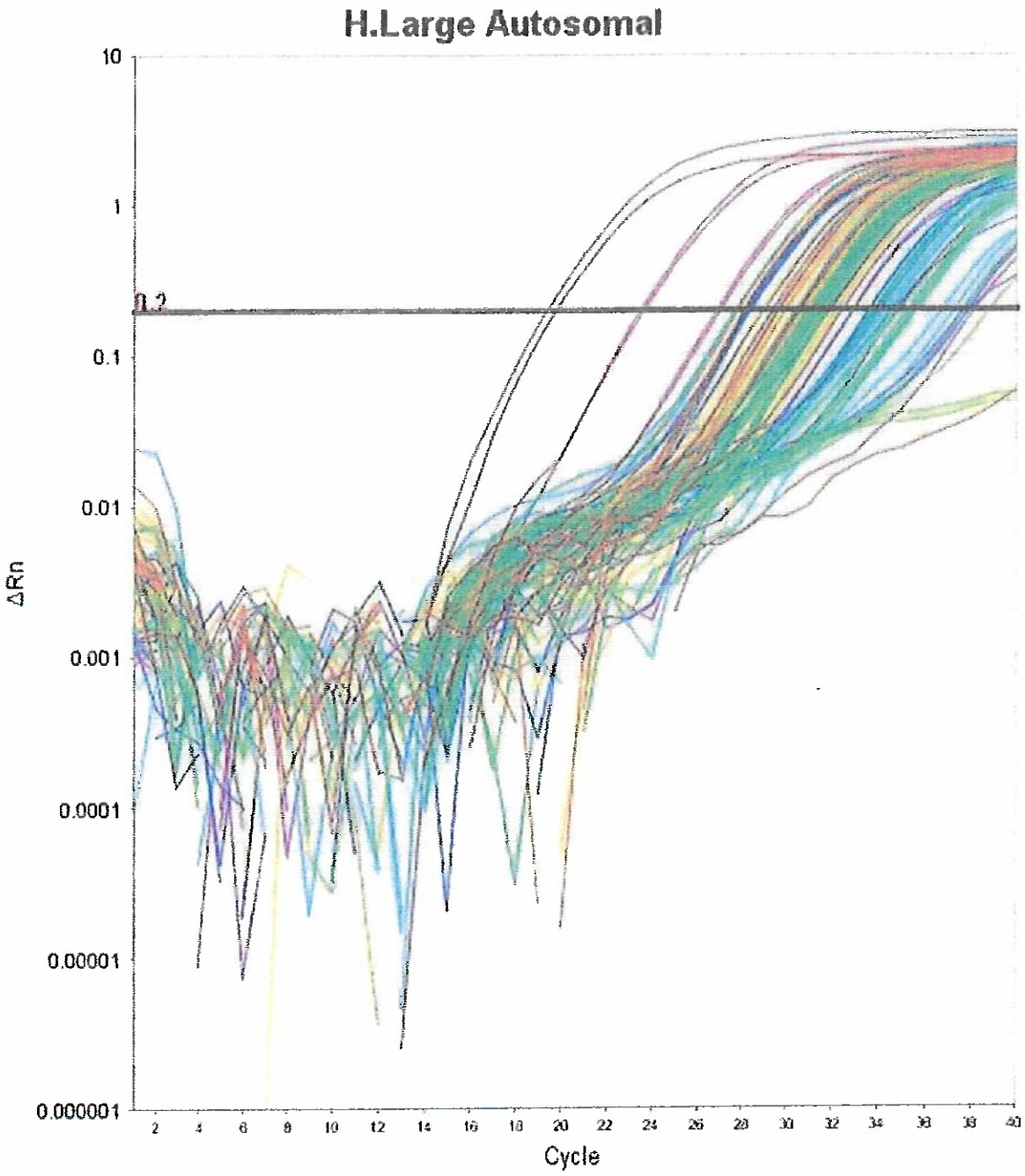
Virtual Standard Curve Summary

Virtual Standard Curve Name	:
Expiration Date	:
Kit Name	:
Target Details	:

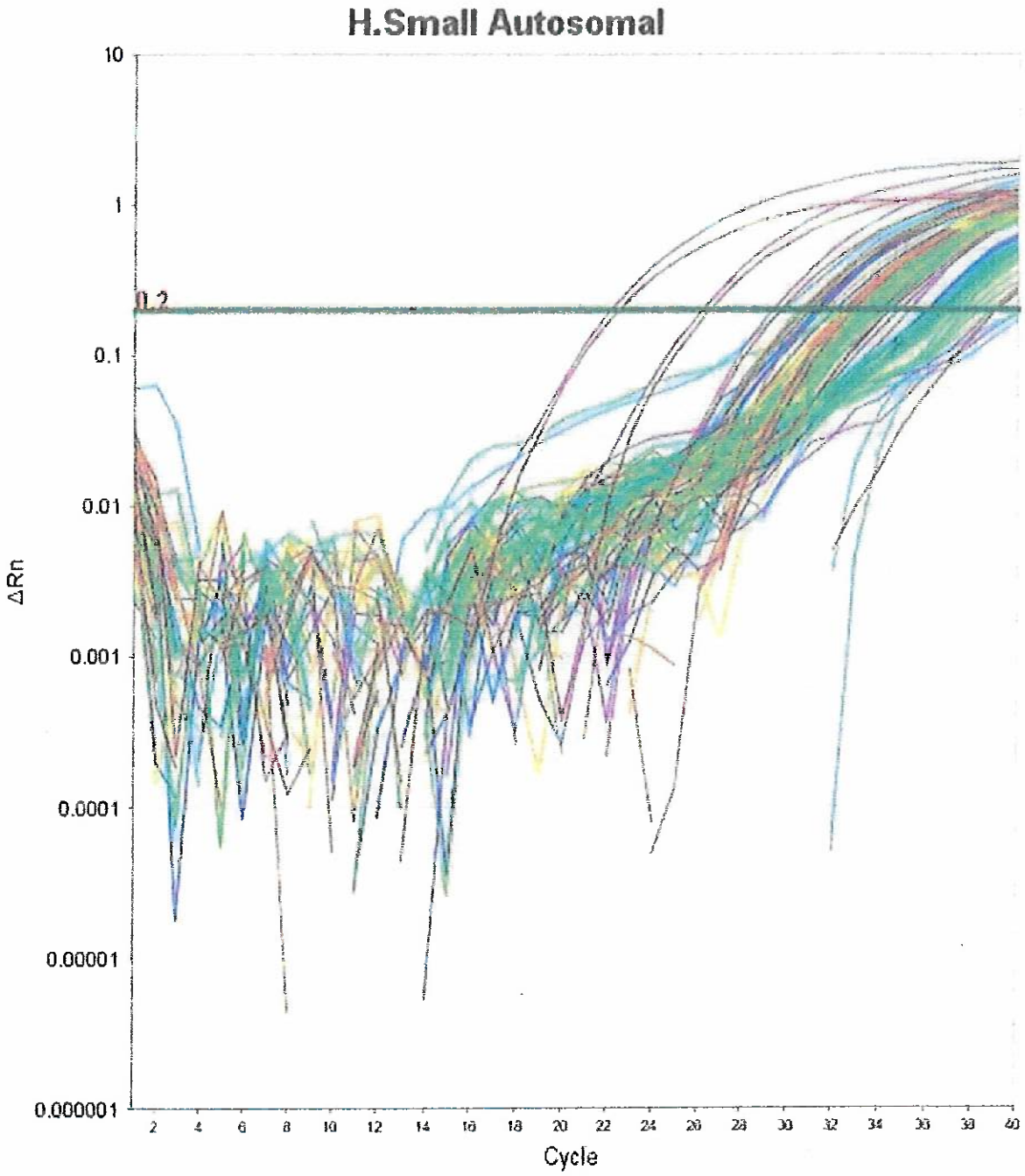
Amplification Plot (ΔRn vs. Cycle)



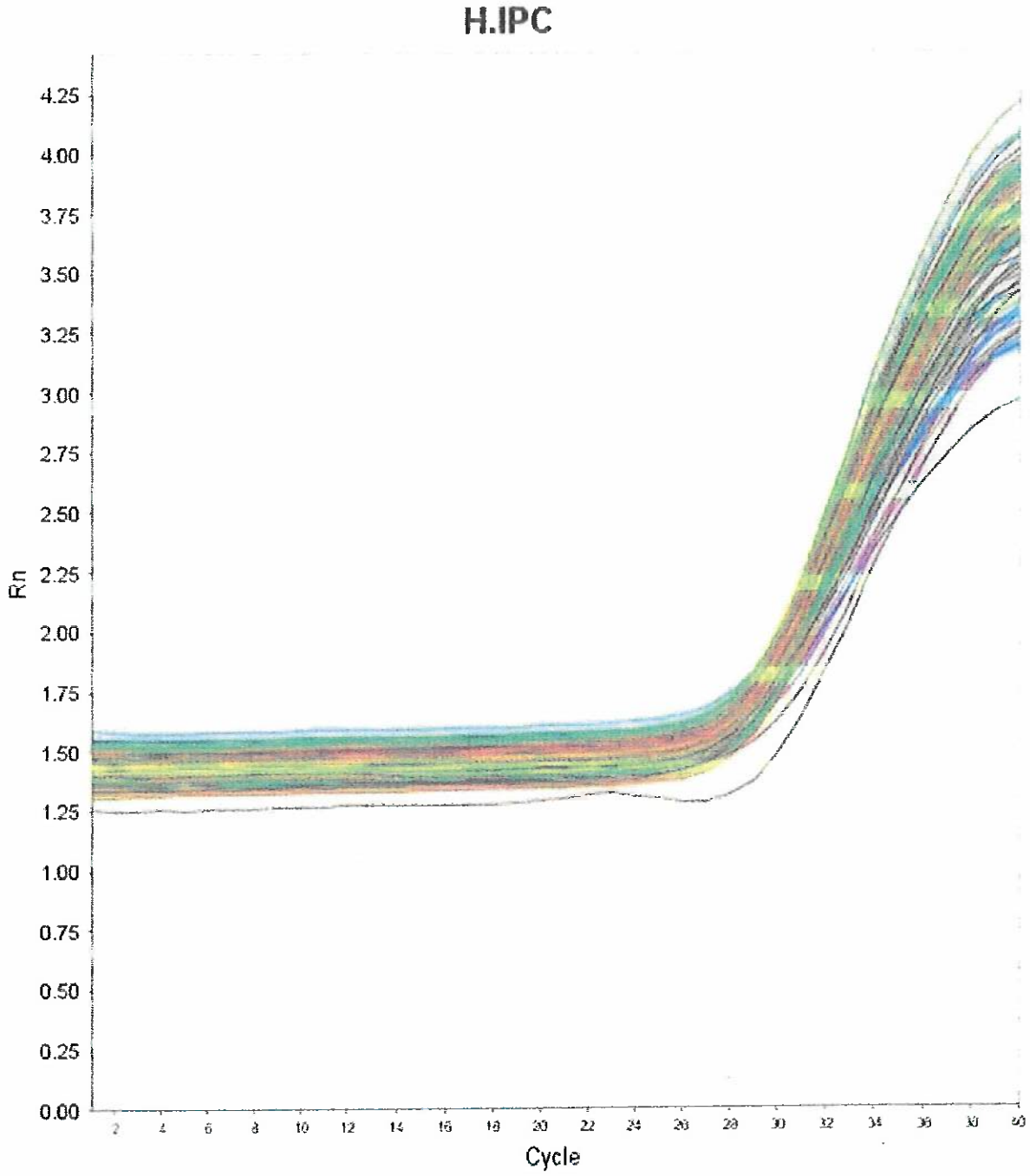
Amplification Plot (ΔRn vs. Cycle)



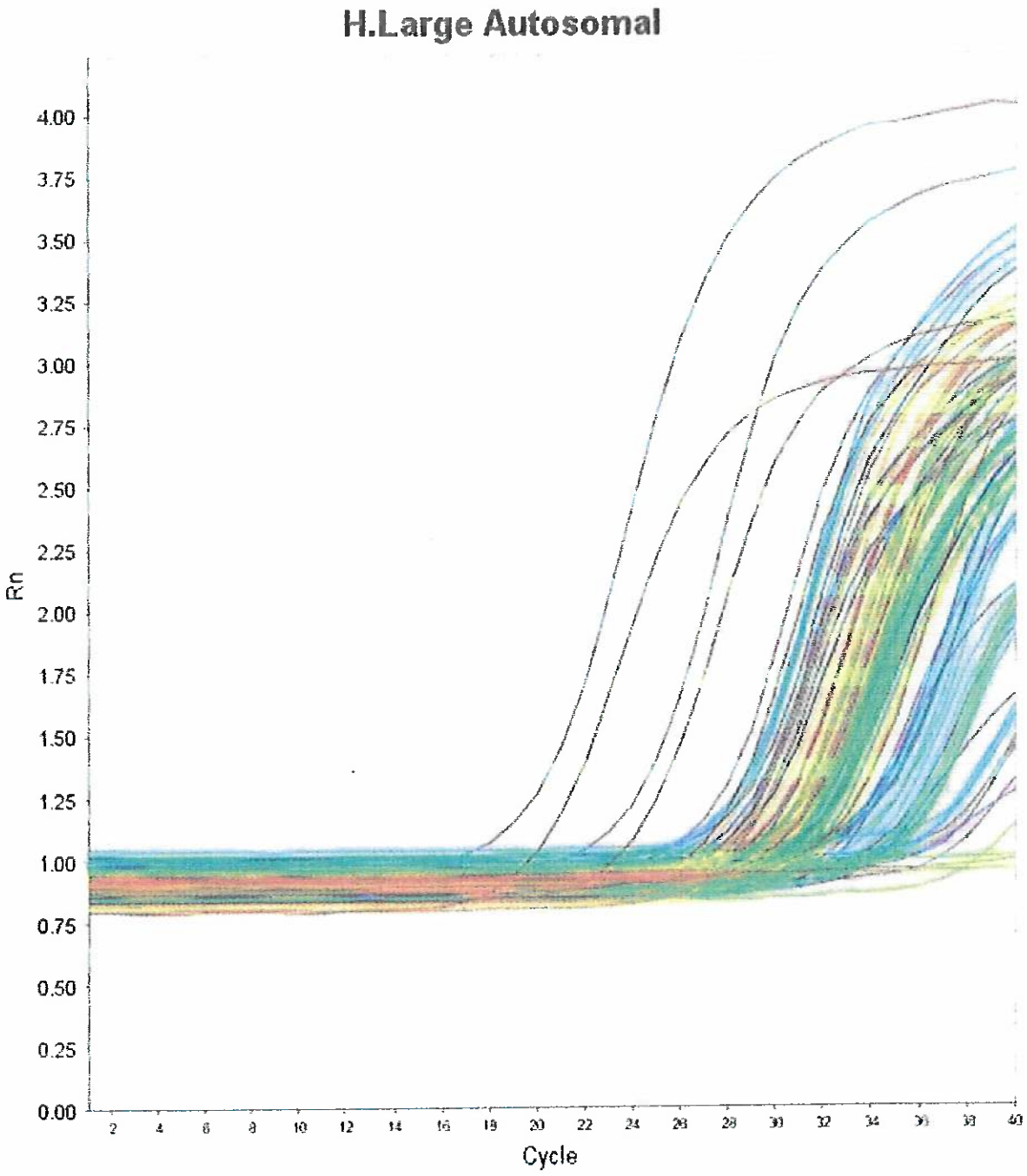
Amplification Plot (ΔRn vs. Cycle)



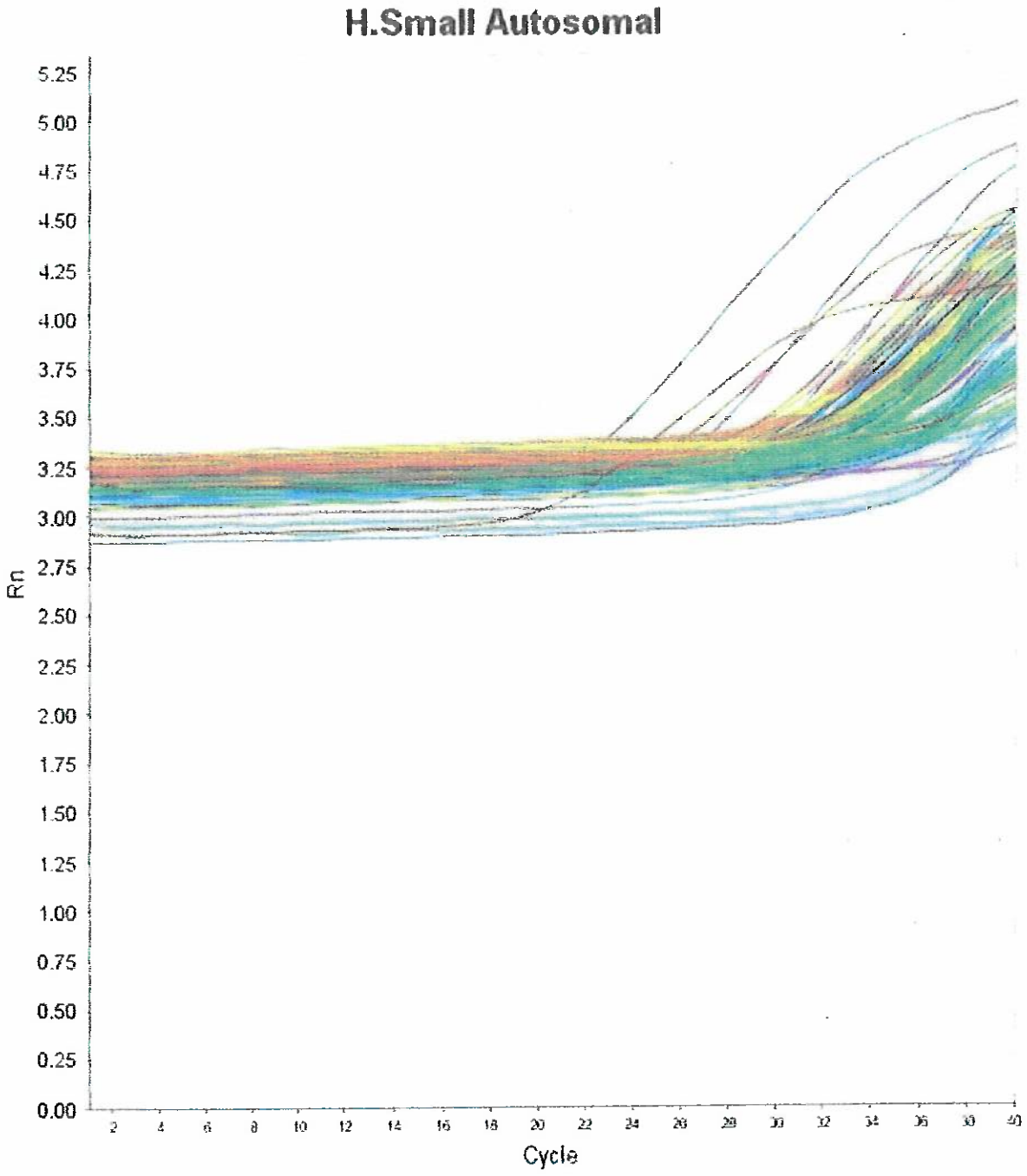
Amplification Plot (Rn vs. Cycle)



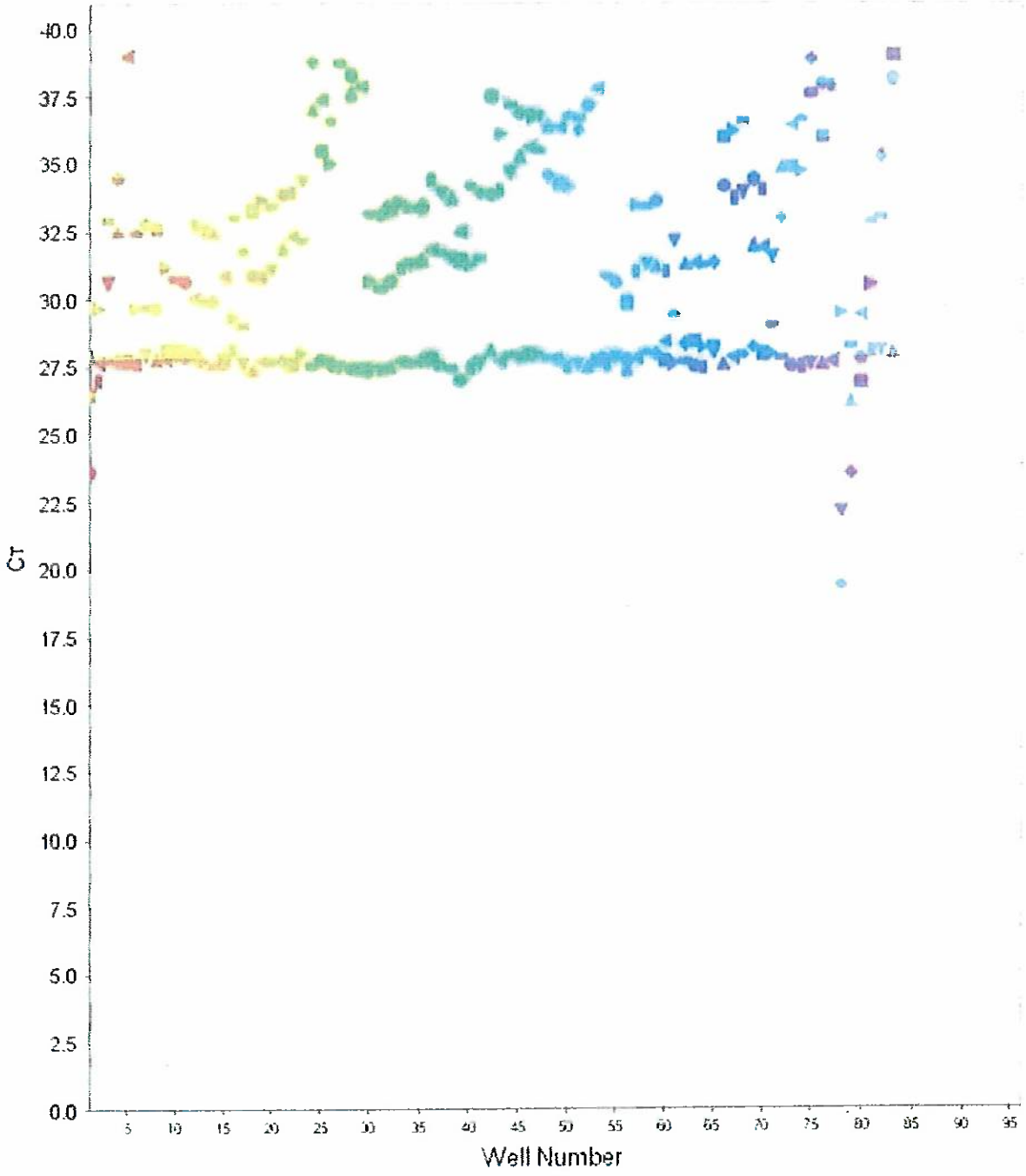
Amplification Plot (Rn vs. Cycle)



Amplification Plot (Rn vs. Cycle)



Amplification Plot (CT vs. Well)



Results Table

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
A1	QHP Standard 1	H.IPC	Unk				29.3255	29.39	0.09
A1	QHP Standard 1	H.Large Autosomal	Std	50			19.7474	19.56	0.27
A1	QHP Standard 1	H.Small Autosomal	Std	50			22.4285	22.26	0.24
A2	QHP Standard 2	H.IPC	Unk				28.0274	28.15	0.17
A2	QHP Standard 2	H.Large Autosomal	Std	5			23.6173	23.55	0.09
A2	QHP Standard 2	H.Small Autosomal	Std	5			26.4753	26.31	0.23
A3	QHP Standard 3	H.IPC	Unk				27.6847	27.73	0.06
A3	QHP Standard 3	H.Large Autosomal	Std	0.5			27.0051	26.93	0.11
A3	QHP Standard 3	H.Small Autosomal	Std	0.5			29.7008	29.54	0.23
A4	QHP Standard 4	H.IPC	Unk				27.756	27.92	0.23
A4	QHP Standard 4	H.Large Autosomal	Std	0.05			30.6228	30.56	0.09
A4	QHP Standard 4	H.Small Autosomal	Std	0.05			32.9388	32.90	0.06
A5	QHP Standard 5	H.IPC	Unk				27.7528	27.91	0.22
A5	QHP Standard 5	H.Large Autosomal	Std	0.005			32.5344	32.76	0.32
A5	QHP Standard 5	H.Small Autosomal	Std	0.005			34.5084	34.87	0.52
A6	NTC	H.IPC	Unk				27.7519	27.88	0.18
A6	NTC	H.Large Autosomal	NTC				Undetermined		
A6	NTC	H.Small Autosomal	NTC				39.0226		
A7	1a	H.IPC	Unk				27.6179	27.80	0.17
A7	1a	H.Large Autosomal	Unk	0.0635	0.06	0.00	29.6653	29.68	0.02
A7	1a	H.Small Autosomal	Unk	0.0459	0.04	0.01	32.4765	32.65	0.18
A8	1a	H.IPC	Unk				27.963	27.80	0.17
A8	1a	H.Large Autosomal	Unk	0.0615	0.06	0.00	29.7111	29.68	0.02
A8	1a	H.Small Autosomal	Unk	0.0354	0.04	0.01	32.8341	32.65	0.18
A9	1a	H.IPC	Unk				27.823	27.80	0.17

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
A9	1a	H.Large Autosomal	Unk	0.063	0.06	0.00	29.6758	29.68	0.02
A9	1a	H.Small Autosomal	Unk	0.0408	0.04	0.01	32.6377	32.65	0.18
A10	2a	H.IPC	Unk				27.78	27.83	0.05
A10	2a	H.Large Autosomal	Unk	0.1855	0.18	0.00	28.109	28.12	0.04
A10	2a	H.Small Autosomal	Unk	0.1169	0.15	0.03	31.1844	30.86	0.28
A11	2a	H.IPC	Unk				27.8778	27.83	0.05
A11	2a	H.Large Autosomal	Unk	0.1875	0.18	0.00	28.0937	28.12	0.04
A11	2a	H.Small Autosomal	Unk	0.1617	0.15	0.03	30.7357	30.86	0.28
A12	2a	H.IPC	Unk				27.8362	27.83	0.05
A12	2a	H.Large Autosomal	Unk	0.179	0.18	0.00	28.1613	28.12	0.04
A12	2a	H.Small Autosomal	Unk	0.17	0.15	0.03	30.6665	30.86	0.28
B1	3a	H.IPC	Unk				27.9652	27.74	0.22
B1	3a	H.Large Autosomal	Unk	0.0492	0.05	0.00	30.0358	29.96	0.06
B1	3a	H.Small Autosomal	Unk	0.038	0.04	0.00	32.7387	32.59	0.13
B2	3a	H.IPC	Unk				27.7202	27.74	0.22
B2	3a	H.Large Autosomal	Unk	0.0524	0.05	0.00	29.9426	29.96	0.06
B2	3a	H.Small Autosomal	Unk	0.0437	0.04	0.00	32.5452	32.59	0.13
B3	3a	H.IPC	Unk				27.5224	27.74	0.22
B3	3a	H.Large Autosomal	Unk	0.0535	0.05	0.00	29.9142	29.96	0.06
B3	3a	H.Small Autosomal	Unk	0.0456	0.04	0.00	32.4863	32.59	0.13
B4	4a	H.IPC	Unk				27.6337	27.79	0.30
B4	4a	H.Large Autosomal	Unk	0.2289	0.14	0.08	27.8041	28.72	0.81
B4	4a	H.Small Autosomal	Unk	0.1477	0.08	0.06	30.861	31.90	1.09
B5	4a	H.IPC	Unk				28.1271	27.79	0.30
B5	4a	H.Large Autosomal	Unk	0.0793	0.14	0.08	29.3416	28.72	0.81
B5	4a	H.Small Autosomal	Unk	0.0307	0.08	0.06	33.0323	31.90	1.09

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
B6	4a	H.IPC	Unk				27.5981	27.79	0.30
B6	4a	H.Large Autosomal	Unk	0.1	0.14	0.08	29.0062	28.72	0.81
B6	4a	H.Small Autosomal	Unk	0.0748	0.08	0.06	31.8002	31.90	1.09
B7	5a	H.IPC	Unk				27.3686	27.58	0.18
B7	5a	H.Large Autosomal	Unk	0.0281	0.03	0.00	30.8492	30.91	0.16
B7	5a	H.Small Autosomal	Unk	0.0254	0.02	0.00	33.2915	33.47	0.20
B8	5a	H.IPC	Unk				27.6959	27.58	0.18
B8	5a	H.Large Autosomal	Unk	0.029	0.03	0.00	30.7992	30.91	0.16
B8	5a	H.Small Autosomal	Unk	0.0192	0.02	0.00	33.6799	33.47	0.20
B9	5a	H.IPC	Unk				27.6722	27.58	0.18
B9	5a	H.Large Autosomal	Unk	0.0238	0.03	0.00	31.0911	30.91	0.16
B9	5a	H.Small Autosomal	Unk	0.0226	0.02	0.00	33.453	33.47	0.20
B10	6a	H.IPC	Unk				27.7383	27.78	0.19
B10	6a	H.Large Autosomal	Unk	0.0137	0.01	0.00	31.8854	32.13	0.22
B10	6a	H.Small Autosomal	Unk	0.0168	0.01	0.00	33.8674	34.07	0.28
B11	6a	H.IPC	Unk				27.6043	27.78	0.19
B11	6a	H.Large Autosomal	Unk	0.0101	0.01	0.00	32.326	32.13	0.22
B11	6a	H.Small Autosomal	Unk	0.0159	0.01	0.00	33.9421	34.07	0.28
B12	6a	H.IPC	Unk				27.9875	27.78	0.19
B12	6a	H.Large Autosomal	Unk	0.0113	0.01	0.00	32.1693	32.13	0.22
B12	6a	H.Small Autosomal	Unk	0.0115	0.01	0.00	34.3918	34.07	0.28
C1	7a	H.IPC	Unk				27.5593	27.64	0.11
C1	7a	H.Large Autosomal	Unk	0.0001	0.00	0.00	38.7801	36.43	2.06
C1	7a	H.Small Autosomal	Unk	0.0017	0.00	0.00	37.0195	37.00	0.39
C2	7a	H.IPC	Unk				27.7622	27.64	0.11
C2	7a	H.Large Autosomal	Unk	0.0011	0.00	0.00	35.5107	36.43	2.06

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	Ct	Ct (Mean)	Ct (Std Dev)
C2	7a	H.Small Autosomal	Unk	0.0013	0.00	0.00	37.3679	37.00	0.39
C3	7a	H.IPC	Unk				27.5965	27.64	0.11
C3	7a	H.Large Autosomal	Unk	0.0016	0.00	0.00	34.9883	36.43	2.06
C3	7a	H.Small Autosomal	Unk	0.0023	0.00	0.00	36.5981	37.00	0.39
C4	8a	H.IPC	Unk				27.5102	27.46	0.04
C4	8a	H.Large Autosomal	Unk				Undetermined	37.63	
C4	8a	H.Small Autosomal	Unk	0.0005	0.00	0.00	38.755	38.31	0.44
C5	8a	H.IPC	Unk				27.4476	27.46	0.04
C5	8a	H.Large Autosomal	Unk	0.0003	0.00		37.6303	37.63	
C5	8a	H.Small Autosomal	Unk	0.0007	0.00	0.00	38.308	38.31	0.44
C6	8a	H.IPC	Unk				27.4241	27.46	0.04
C6	8a	H.Large Autosomal	Unk				Undetermined	37.63	
C6	8a	H.Small Autosomal	Unk	0.0009	0.00	0.00	37.8758	38.31	0.44
C7	1b	H.IPC	Unk				27.3845	27.42	0.07
C7	1b	H.Large Autosomal	Unk	0.0321	0.03	0.00	30.6548	30.57	0.15
C7	1b	H.Small Autosomal	Unk	0.0277	0.03	0.00	33.1726	33.21	0.14
C8	1b	H.IPC	Unk				27.3697	27.42	0.07
C8	1b	H.Large Autosomal	Unk	0.0383	0.03	0.00	30.399	30.57	0.15
C8	1b	H.Small Autosomal	Unk	0.0294	0.03	0.00	33.0911	33.21	0.14
C9	1b	H.IPC	Unk				27.5024	27.42	0.07
C9	1b	H.Large Autosomal	Unk	0.0319	0.03	0.00	30.6636	30.57	0.15
C9	1b	H.Small Autosomal	Unk	0.0243	0.03	0.00	33.356	33.21	0.14
C10	2b	H.IPC	Unk				27.6969	27.69	0.05
C10	2b	H.Large Autosomal	Unk	0.0231	0.02	0.00	31.1324	31.29	0.16
C10	2b	H.Small Autosomal	Unk	0.0209	0.02	0.00	33.565	33.44	0.11
C11	2b	H.IPC	Unk				27.6367	27.69	0.05

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
C11	2b	H.Large Autosomal	Unk	0.0207	0.02	0.00	31.2899	31.29	0.16
C11	2b	H.Small Autosomal	Unk	0.0245	0.02	0.00	33.3445	33.44	0.11
C12	2b	H.IPC	Unk				27.7396	27.69	0.05
C12	2b	H.Large Autosomal	Unk	0.0184	0.02	0.00	31.4585	31.29	0.16
C12	2b	H.Small Autosomal	Unk	0.0233	0.02	0.00	33.4152	33.44	0.11
D1	3b	H.IPC	Unk				27.8206	27.63	0.17
D1	3b	H.Large Autosomal	Unk	0.0143	0.02	0.00	31.8276	31.71	0.13
D1	3b	H.Small Autosomal	Unk	0.0109	0.01	0.00	34.4599	34.06	0.36
D2	3b	H.IPC	Unk				27.5985	27.63	0.17
D2	3b	H.Large Autosomal	Unk	0.0153	0.02	0.00	31.7268	31.71	0.13
D2	3b	H.Small Autosomal	Unk	0.0158	0.01	0.00	33.9503	34.06	0.36
D3	3b	H.IPC	Unk				27.4808	27.63	0.17
D3	3b	H.Large Autosomal	Unk	0.0172	0.02	0.00	31.5615	31.71	0.13
D3	3b	H.Small Autosomal	Unk	0.0179	0.01	0.00	33.7766	34.06	0.36
D4	4b	H.IPC	Unk				27.0185	27.36	0.30
D4	4b	H.Large Autosomal	Unk	0.0183	0.02	0.00	31.4698	31.42	0.13
D4	4b	H.Small Autosomal	Unk	0.045	0.02	0.02	32.5022	33.55	0.92
D5	4b	H.IPC	Unk				27.4621	27.36	0.30
D5	4b	H.Large Autosomal	Unk	0.0211	0.02	0.00	31.2656	31.42	0.13
D5	4b	H.Small Autosomal	Unk	0.0131	0.02	0.02	34.2102	33.55	0.92
D6	4b	H.IPC	Unk				27.5995	27.36	0.30
D6	4b	H.Large Autosomal	Unk	0.0177	0.02	0.00	31.52	31.42	0.13
D6	4b	H.Small Autosomal	Unk	0.0159	0.02	0.02	33.9368	33.55	0.92
D7	5b	H.IPC	Unk				28.1283	27.93	0.21
D7	5b	H.Large Autosomal	Unk	0.0035	0.00	0.00	33.8705	34.20	0.43
D7	5b	H.Small Autosomal	Unk	0.0012	0.00	0.00	37.5371	36.96	0.74

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	Ct	Ct (Mean)	Ct (Std Dev)
D8	5b	H.IPC	Unk				27.708	27.93	0.21
D8	5b	H.Large Autosomal	Unk	0.0031	0.00	0.00	34.0431	34.20	0.43
D8	5b	H.Small Autosomal	Unk	0.0033	0.00	0.00	36.1324	36.96	0.74
D9	5b	H.IPC	Unk				27.9624	27.93	0.21
D9	5b	H.Large Autosomal	Unk	0.002	0.00	0.00	34.689	34.20	0.43
D9	5b	H.Small Autosomal	Unk	0.0015	0.00	0.00	37.214	36.96	0.74
D10	6b	H.IPC	Unk				27.8346	27.92	0.07
D10	6b	H.Large Autosomal	Unk	0.0013	0.00	0.00	35.3243	35.50	0.16
D10	6b	H.Small Autosomal	Unk	0.0019	0.00	0.00	36.9039	36.82	0.07
D11	6b	H.IPC	Unk				27.9579	27.92	0.07
D11	6b	H.Large Autosomal	Unk	0.001	0.00	0.00	35.6455	35.50	0.16
D11	6b	H.Small Autosomal	Unk	0.002	0.00	0.00	36.7875	36.82	0.07
D12	6b	H.IPC	Unk				27.9697	27.92	0.07
D12	6b	H.Large Autosomal	Unk	0.0011	0.00	0.00	35.5223	35.50	0.16
D12	6b	H.Small Autosomal	Unk	0.0021	0.00	0.00	36.7716	36.82	0.07
E1	7b	H.IPC	Unk				27.7988	27.69	0.16
E1	7b	H.Large Autosomal	Unk	0.0022	0.00	0.00	34.5623	34.34	0.21
E1	7b	H.Small Autosomal	Unk	0.0026	0.00	0.00	36.4502	36.54	0.22
E2	7b	H.IPC	Unk				27.7773	27.69	0.16
E2	7b	H.Large Autosomal	Unk	0.0026	0.00	0.00	34.3239	34.34	0.21
E2	7b	H.Small Autosomal	Unk	0.0027	0.00	0.00	36.37	36.54	0.22
E3	7b	H.IPC	Unk				27.5051	27.69	0.16
E3	7b	H.Large Autosomal	Unk	0.0029	0.00	0.00	34.1446	34.34	0.21
E3	7b	H.Small Autosomal	Unk	0.002	0.00	0.00	36.7916	36.54	0.22
E4	8b	H.IPC	Unk				27.5768	27.60	0.10
E4	8b	H.Large Autosomal	Unk	0.0005	0.00	0.00	36.6903	37.22	0.55

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
E4	8b	H.Small Autosomal	Unk	0.003	0.00		36.2473	36.25	
E5	8b	H.IPC	Unk				27.5128	27.60	0.10
E5	8b	H.Large Autosomal	Unk	0.0004	0.00	0.00	37.1762	37.22	0.55
E5	8b	H.Small Autosomal	Unk				Undetermined	36.25	
E6	8b	H.IPC	Unk				27.7103	27.60	0.10
E6	8b	H.Large Autosomal	Unk	0.0002	0.00	0.00	37.7882	37.22	0.55
E6	8b	H.Small Autosomal	Unk				Undetermined	36.25	
E7	1c	H.IPC	Unk				27.5986	27.60	0.26
E7	1c	H.Large Autosomal	Unk	0.2261	0.23	0.01	27.8222	27.80	0.06
E7	1c	H.Small Autosomal	Unk	0.1504	0.21	0.08	30.8353	30.44	0.52
E8	1c	H.IPC	Unk				27.864	27.60	0.26
E8	1c	H.Large Autosomal	Unk	0.2226	0.23	0.01	27.8447	27.80	0.06
E8	1c	H.Small Autosomal	Unk	0.1758	0.21	0.08	30.6197	30.44	0.52
E9	1c	H.IPC	Unk				27.3421	27.60	0.26
E9	1c	H.Large Autosomal	Unk	0.2394	0.23	0.01	27.7391	27.80	0.06
E9	1c	H.Small Autosomal	Unk	0.3066	0.21	0.08	29.8514	30.44	0.52
E10	2c	H.IPC	Unk				27.813	27.92	0.10
E10	2c	H.Large Autosomal	Unk	0.0248	0.02	0.00	31.027	31.18	0.13
E10	2c	H.Small Autosomal	Unk	0.0226	0.02	0.00	33.4574	33.50	0.10
E11	2c	H.IPC	Unk				27.9999	27.92	0.10
E11	2c	H.Large Autosomal	Unk	0.0208	0.02	0.00	31.2853	31.18	0.13
E11	2c	H.Small Autosomal	Unk	0.0231	0.02	0.00	33.4225	33.50	0.10
E12	2c	H.IPC	Unk				27.9583	27.92	0.10
E12	2c	H.Large Autosomal	Unk	0.0217	0.02	0.00	31.2205	31.18	0.13
E12	2c	H.Small Autosomal	Unk	0.0201	0.02	0.00	33.6161	33.50	0.10
F1	3c	H.IPC	Unk				27.7311	27.67	0.06

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
F1	3c	H.Large Autosomal	Unk	0.149	0.13	0.05	28.4269	28.72	0.61
F1	3c	H.Small Autosomal	Unk	0.1334	0.10	0.04	31.0015	31.46	0.58
F2	3c	H.IPC	Unk				27.6063	27.67	0.06
F2	3c	H.Large Autosomal	Unk	0.075	0.13	0.05	29.4231	28.72	0.61
F2	3c	H.Small Autosomal	Unk	0.0599	0.10	0.04	32.1083	31.46	0.58
F3	3c	H.IPC	Unk				27.6709	27.67	0.06
F3	3c	H.Large Autosomal	Unk	0.1613	0.13	0.05	28.3125	28.72	0.61
F3	3c	H.Small Autosomal	Unk	0.1098	0.10	0.04	31.2705	31.46	0.58
F4	4c	H.IPC	Unk				27.4901	27.63	0.31
F4	4c	H.Large Autosomal	Unk	0.1515	0.16	0.01	28.403	28.31	0.09
F4	4c	H.Small Autosomal	Unk	0.106	0.11	0.00	31.3197	31.30	0.04
F5	4c	H.IPC	Unk				27.4218	27.63	0.31
F5	4c	H.Large Autosomal	Unk	0.1718	0.16	0.01	28.2206	28.31	0.09
F5	4c	H.Small Autosomal	Unk	0.1111	0.11	0.00	31.254	31.30	0.04
F6	4c	H.IPC	Unk				27.9842	27.63	0.31
F6	4c	H.Large Autosomal	Unk	0.1617	0.16	0.01	28.3087	28.31	0.09
F6	4c	H.Small Autosomal	Unk	0.1055	0.11	0.00	31.3256	31.30	0.04
F7	5c	H.IPC	Unk				27.5515	27.77	0.19
F7	5c	H.Large Autosomal	Unk	0.0029	0.00	0.00	34.1668	33.93	0.23
F7	5c	H.Small Autosomal	Unk	0.0036	0.00	0.00	35.9855	36.27	0.30
F8	5c	H.IPC	Unk				27.8241	27.77	0.19
F8	5c	H.Large Autosomal	Unk	0.0039	0.00	0.00	33.7082	33.93	0.23
F8	5c	H.Small Autosomal	Unk	0.003	0.00	0.00	36.2272	36.27	0.30
F9	5c	H.IPC	Unk				27.9233	27.77	0.19
F9	5c	H.Large Autosomal	Unk	0.0034	0.00	0.00	33.922	33.93	0.23
F9	5c	H.Small Autosomal	Unk	0.0023	0.00	0.00	36.5885	36.27	0.30

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	Ct	Ct (Mean)	Ct (Std Dev)
F10	6c	H.IPC	Unk				28.2	28.03	0.15
F10	6c	H.Large Autosomal	Unk	0.0131	0.04	0.05	31.953	30.95	1.68
F10	6c	H.Small Autosomal	Unk	0.0113	0.04	0.04	34.4125	33.33	1.57
F11	6c	H.IPC	Unk				27.9469	28.03	0.15
F11	6c	H.Large Autosomal	Unk	0.0138	0.04	0.05	31.882	30.95	1.68
F11	6c	H.Small Autosomal	Unk	0.0148	0.04	0.04	34.0423	33.33	1.57
F12	6c	H.IPC	Unk				27.9504	28.03	0.15
F12	6c	H.Large Autosomal	Unk	0.0998	0.04	0.05	29.0081	30.95	1.68
F12	6c	H.Small Autosomal	Unk	0.0907	0.04	0.04	31.5343	33.33	1.57
G1	7c	H.IPC	Unk				27.8135	27.59	0.20
G1	7c	H.Large Autosomal	Unk	0.0065	0.00	0.00	32.9789	34.19	1.05
G1	7c	H.Small Autosomal	Unk	0.0082	0.00	0.00	34.8597	35.97	0.97
G2	7c	H.IPC	Unk				27.4785	27.59	0.20
G2	7c	H.Large Autosomal	Unk	0.0018	0.00	0.00	34.8384	34.19	1.05
G2	7c	H.Small Autosomal	Unk	0.0026	0.00	0.00	36.4262	35.97	0.97
G3	7c	H.IPC	Unk				27.4648	27.59	0.20
G3	7c	H.Large Autosomal	Unk	0.0019	0.00	0.00	34.7426	34.19	1.05
G3	7c	H.Small Autosomal	Unk	0.0023	0.00	0.00	36.6252	35.97	0.97
G4	8c	H.IPC	Unk				27.5556	27.60	0.06
G4	8c	H.Large Autosomal	Unk	0.0003	0.00	0.00	37.5967	37.80	0.18
G4	8c	H.Small Autosomal	Unk	0.0005	0.00	0.00	38.8526	37.42	2.02
G5	8c	H.IPC	Unk				27.5747	27.60	0.06
G5	8c	H.Large Autosomal	Unk	0.0002	0.00	0.00	37.9447	37.80	0.18
G5	8c	H.Small Autosomal	Unk	0.0036	0.00	0.00	35.9935	37.42	2.02
G6	8c	H.IPC	Unk				27.6646	27.60	0.06
G6	8c	H.Large Autosomal	Unk	0.0002	0.00	0.00	37.8705	37.80	0.18

Well	Sample	Target	Task	Quantity	Quantity (Mean)	Quantity (Std Dev)	CT	CT (Mean)	CT (Std Dev)
G6	8c	H.Small Autosomal	Unk				Undetermined	37.42	2.02
G7	QHP Standard 1	H.IPC	Unk				29.4483	29.39	0.09
G7	QHP Standard 1	H.Large Autosomal	Std	50			19.3716	19.56	0.27
G7	QHP Standard 1	H.Small Autosomal	Std	50			22.0847	22.26	0.24
G8	QHP Standard 2	H.IPC	Unk				28.2653	28.15	0.17
G8	QHP Standard 2	H.Large Autosomal	Std	5			23.4852	23.55	0.09
G8	QHP Standard 2	H.Small Autosomal	Std	5			26.1447	26.31	0.23
G9	QHP Standard 3	H.IPC	Unk				27.7766	27.73	0.06
G9	QHP Standard 3	H.Large Autosomal	Std	0.5			26.8481	26.93	0.11
G9	QHP Standard 3	H.Small Autosomal	Std	0.5			29.3741	29.54	0.23
G10	QHP Standard 4	H.IPC	Unk				28.0785	27.92	0.23
G10	QHP Standard 4	H.Large Autosomal	Std	0.05			30.4897	30.56	0.09
G10	QHP Standard 4	H.Small Autosomal	Std	0.05			32.8608	32.90	0.06
G11	QHP Standard 5	H.IPC	Unk				28.0683	27.91	0.22
G11	QHP Standard 5	H.Large Autosomal	Std	0.005			32.9863	32.76	0.32
G11	QHP Standard 5	H.Small Autosomal	Std	0.005			35.2386	34.87	0.52
G12	NTC	H.IPC	Unk				28.0025	27.88	0.18
G12	NTC	H.Large Autosomal	NTC				38.131		
G12	NTC	H.Small Autosomal	NTC				38.9992		

QC Summary

Total Wells	96	Processed Wells	84	Targets Used	3
Well Setup	84	Flagged Wells	46	Samples Used	30

Flag	Name	Frequency	Locations
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CTFAIL	C_T algorithm failed	0	
DRNMIN	Define acceptable delta Rn based on C_T range	0	
EXPFAIL	Exponential algorithm failed	2	C4, C6
HIGHQT	High Quantity of DNA	0	
HIGHSD	High standard deviation in replicate group	31	A5, B4, B5, B6, C1, C2, C3, D4, D5, D6, D7, D8, D9, E4, E5, E6, E7, E8, E9, F1, F2, F3, F10, F11, F12, G1, G2, G3, G4, G5, G11
IPCCT	Internal PCR Control C_T value	0	
LOWQT	Low Quantity of DNA	27	C1, C2, C3, C4, C5, C6, D7, D8, D9, D10, D11, D12, E1, E2, E3, E4, E5, E6, F7, F8, F9, G1, G2, G3, G4, G5, G6
NOAMP	No amplification	0	
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
NTCCT	Non-Template Control sample amplification	2	A6, G12
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
PRFDROP	Passive reference signal changes near C_T	0	
PRFLOW	Low passive reference signal	0	
R^2	Low Standard curve R^2 value	0	
SLOPE	Non-optimal slope of the Standard curve	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	
YINT	Y-Intercept	0	