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The Forensic Value of Processed Human Hair Extensions

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The Forensic Value of Processed Human Hair Extensions

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Abstract

The human hair extension industry has grown immensely with revenues exceeding nine billion dollars each year. Statistics indicate that over sixty percent of women have at some point invested in hair extensions and that they are even becoming popular with men. In spite of the expansion of the extension consumer market, human hair extensions have never been evaluated for their evidentiary value in a forensic case. A human hair extension collected from a crime scene would be evaluated as a shed human telogen hair and analyzed using microscopic techniques and mitochondrial DNA (mtDNA). Sequencing of mtDNA from the extension would place the hair donor, not the suspect, at the scene. Although it is not likely that the mtDNA sequence would be matched to the donor's maternal lineage, the evidence would be misleading. Hair extensions collected from a crime scene would misdirect an investigation and result in a misuse of time and resources. The ability to identify a human hair as an extension would be invaluable during an investigation and might exclude the hair as probative evidence.

In this study, three brands of processed human hair extensions were evaluated microscopically and genetically for their probative value in forensic casework. Microscopic analysis of hair morphology by transmitted light microscopy and scanning electron microscopy determined that the internal and surface characteristics of the human hair extensions were consistent with human head hair and failed to identify any distinguishing features (pitting, striations, indentations, internal variations) that differentiated the extensions from natural human hair. Chemical analysis by an energy dispersive detector (EDX) in conjunction with an SEM identified carbon, oxygen, sulfur, aluminum, and calcium as the main elemental components of the processed human hair extensions which is consistent with human hair. No elements unique to the extensions were detected.

mtDNA extracted from the hair extensions was sequenced and compared to the revised Cambridge sequence (rCRS) to identify single nucleotide polymorphisms (SNPs). SNPs were used to assign haplotypes and distinguish regional affiliations associated with the extensions in an attempt to establish the ethnicity of the hair donor's maternal lineage. Haplotype assignments for the hair extensions were based on HV2 genetic polymorphisms and represented multiple geographic regions and a large portion of the population. HV2 sequences were not restrictive enough to determine regional affiliations for particular extension brands or processed human hair extensions as a whole. More definitive haplotype assignments would be possible with HV1 SNP discrimination. Also, sequence variation between hair extensions of the same brand indicated that the hair within a single package of extensions was from multiple donors. This has significant implications in forensic analysis.

Keywords Processed Human Hair Extensions, Mitochondrial DNA, Haplotype, Hair Microscopy

Table of Contents

List of Figures

List of Tables

THE FORENSIC VALUE OF PROCESSED HUMAN HAIR EXTENSIONS

Introduction

Hair extensions are a nine billion dollar per year industry with sales increasing thirty-five to fifty percent each year (Chris Rock Entertainment & HBO Films, 2009; Hayt, 2006). Statistics indicate that over sixty percent of women have at some point invested in hair extensions and that ninety-five percent of the market is composed of women ages thirty to seventy (Hayt, 2006). Although hair extensions are most common among African American women, they have become increasingly popular with women of other ethnicities including Caucasian, Latino, and Asian (Dawson, 2009). Reports also indicate that extensions are now being used by men (Ruddock, 2012). Hair extensions made from human head hair are the most frequently purchased form of extension, showing a seventy percent increase in sales over synthetic or animal hair extensions (Dawson, 2009). Until now, human hair extensions have never been investigated for their evidentiary value in a forensic case.

A hair from a human derived hair extension recovered at a crime scene would likely be identified as a shed human telogen hair and processed using microscopic techniques and mitochondrial DNA (mtDNA) analysis. Sequencing of mtDNA from the extension would place the hair donor, not the suspect, at the scene. Although it is not likely that the mtDNA sequence would be matched to the donor's maternal lineage, the evidence would be misleading. Hair extensions collected from a crime scene would misdirect an investigation and result in a misuse of time and resources. The ability to identify a human hair as an extension would be invaluable during an investigation and might exclude the hair as probative evidence. It would also be beneficial to be able to definitively associate a processed human hair extension collected from a crime scene to others from the same manufacturing package, particularly if the extensions were still attached to the suspect's head.

THE FORENSIC VALUE OF PROCESSED HUMAN HAIR EXTENSIONS

In this study, processed human hair extensions were evaluated genetically and microscopically for their probative value in forensic casework. Current extraction techniques and detection technologies were used to recover, quantify, amplify, and sequence mtDNA from processed human hair extensions and to determine if the grade or quality of the hair extensions affected the results. mtDNA was also used to assign haplotypes and indicate the ethnicity of the hair donor's maternal lineage. In addition, this study attempted to differentiate between a processed human hair extension and a natural human head hair based on physical, optical, and chemical attributes. A stereomicroscope, an inverted research microscope, and a scanning electron microscope (SEM) were used to compare the hair morphology of processed human hair extensions to real human head hairs. Chemical analysis using an energy dispersive detector (EDX) in conjunction with the SEM attempted to identify any distinguishing elements associated with the extensions not found in human hair. Human hair extensions were the only forensically relevant type of extension as they could potentially be mistaken for real human head hair. Synthetic and animal hair extensions were not addressed in this study.

Hair Physiology

Hair is a "slender thread-like outgrowth" of dead, keratinized cells. It consists of the hair root which lies underneath the skin and the hair shaft which extends from follicles above the skin surface (Deedrick and Koch, 2004a; Hicks, 1977). The root is enclosed within the follicle and is nourished by the dermal papilla which promotes proliferation and differentiation of cells as they move upward and form

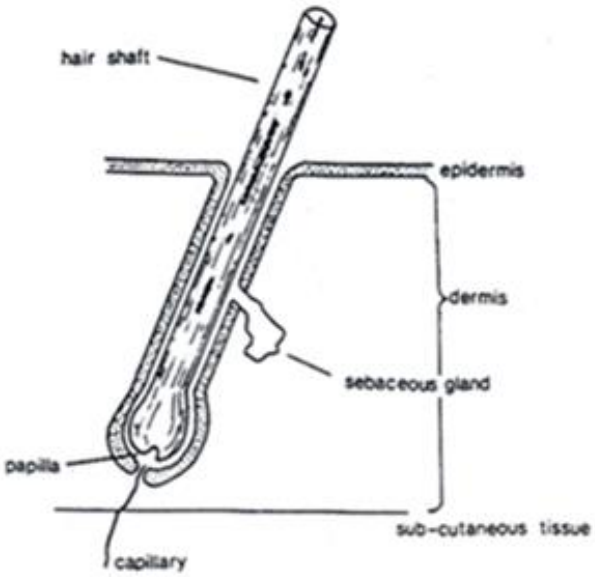


Figure 1: Diagram of hair imbedded in skin. (Deedrick & Koch, 2004a)

the hair shaft (Driskell et *al*., 2011). The hair shaft is composed of keratin, melanin, and small amounts of metallic elements.

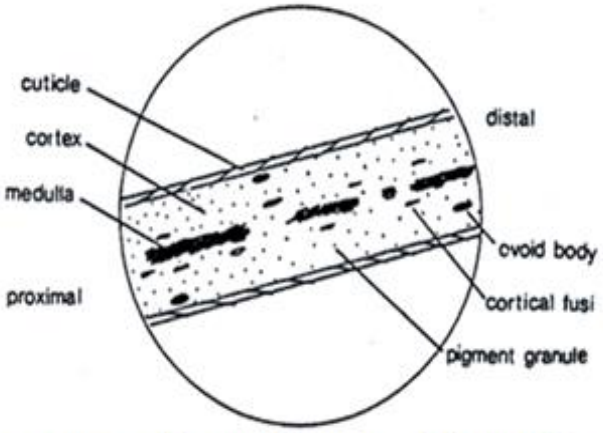


Figure 2: Diagram of the parts of a human hair. (Deedrick & Koch, 2004a)

The hair can be divided into three regions: the cuticle, the cortex and the medulla (Deedrick and Koch, 2004a; Hicks, 1977). The cuticle is the translucent surface of the hair shaft. It is composed of scales that function as a protective layer and run from the proximal or root end of the hair to the distal end or tip. Scale pattern varies by species and assists in the identification of species type during microscopic analysis. There are three general scale patterns: coronal (crown-like), spinous (petal-like), and imbricate. Human hair has an imbricate scale pattern characterized by irregular shaped scales that overlap (Deedrick and Koch, 2004a; Hicks, 1977).

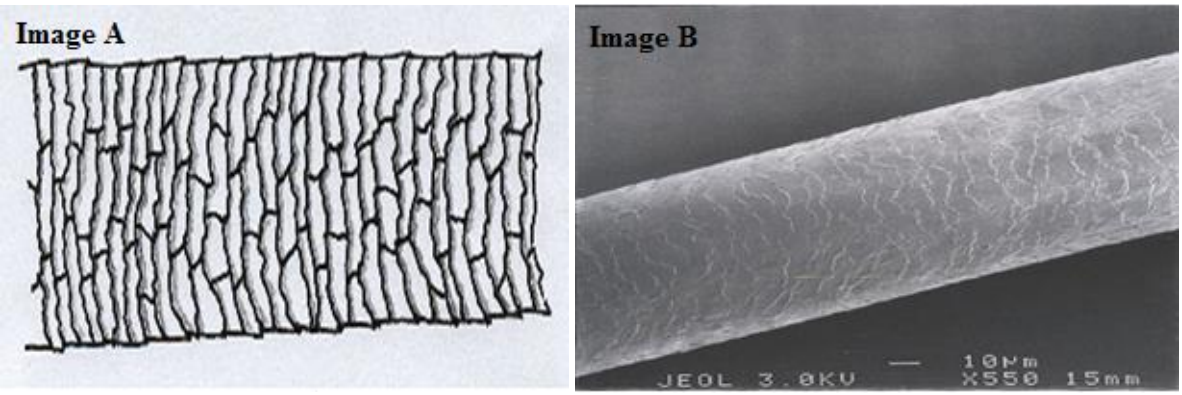


Figure 3 (Deedrick and Koch, 2004a)
Image A: Diagram of imbricate scale pattern.
Image B: Photomicrograph of the surface of a human hair shaft showing imbricate scale pattern.

The cortex consists of "elongated and fusiform cells" and comprises the majority of the hair shaft. It is the internal region between the cuticle and the medulla and can contain cortical fusi, pigment granules, and ovoid bodies (Deedrick and Koch, 2004a; Hicks, 1977). These features can be forensically significant and help to characterize hair (Hicks, 1977). Cortical fusi are airspaces of varying shapes and sizes. They present as small, dark, irregularly shaped particles and are generally found near the proximal end of the hair, although they can be located throughout the length of the hair shaft (Deedrick and Koch, 2004a; Hicks, 1977). During analysis the size, shape, color, density, and distribution of cortical fusi should be considered (Hicks, 1977). Pigment granules are small and dark and have a granular structure. They are smaller than cortical fusi and have varying colors, densities and distributions (Deedrick and Koch, 2004a; Hicks, 1977). Usually pigment granules are distributed toward the cuticle in human hairs (Deedrick and Koch, 2004a). Ovoid bodies are much larger than cortical fusi or pigment granules. They tend to be black with a spherical structure and well-defined margins (Deedrick and Koch, 2004a; Hicks, 1977).

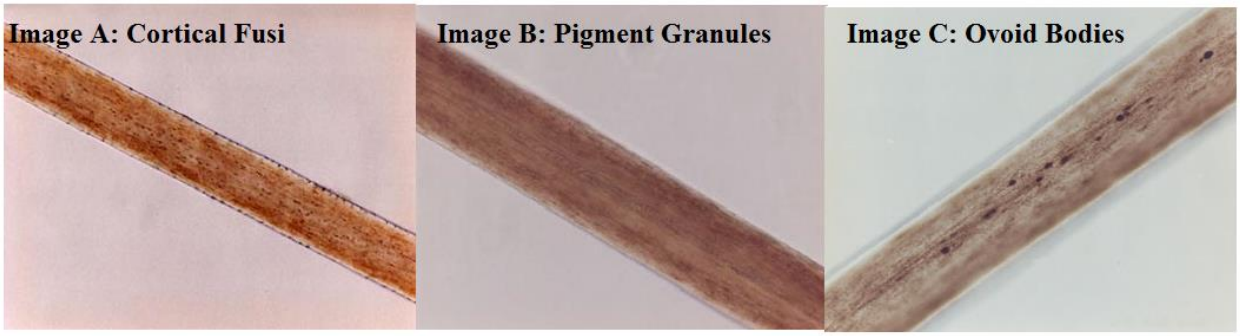


Figure 4 (Deedrick and Koch, 2004a)
Image A: Photomicrograph of cortical fusi in human hair.
Image B: Photomicrograph of pigment distribution in human hair.
Image C: Photomicrograph of ovoid bodies in human hair.

The medulla is a composed of localized cells that run through the center of the hair shaft (Deedrick and Koch, 2004a; Hicks, 1977). This central core of cells can be filled with air causing it to appear black or opaque when examined with transmitted light. Human hairs will generally exhibit an amorphous medulla that is identified as either fragmentary, continuous or discontinuous.

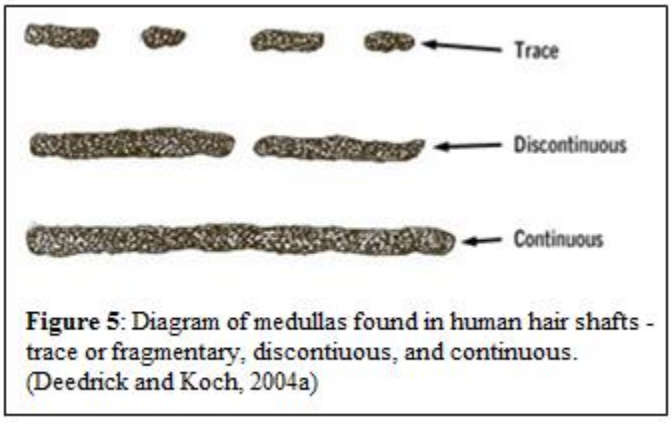


Figure 5: Diagram of medullas found in human hair shafts - trace or fragmentary, discontiuous, and continuous. (Deedrick and Koch, 2004a)

Mitochondrial DNA (mtDNA)

The mitochondria of a eukaryotic cell are located throughout the cytoplasm and produce the vast majority of adenosine triphosphate (ATP) through the process of oxidative phosphorylation (Ingman & Gyllensten, 2001; Smeitink *et al*., 2001). ATP is an essential cellular component as it stores energy necessary for cellular metabolism (Trounce, 2000). The average human cell can contain up to two thousand mitochondria and each mitochondrion has approximately fifteen copies of mitochondrial DNA resulting in a single cell having thousands of copies of the mitochondrial genome (Cavelier *et al.*, 2000). The circular mtDNA genome is double stranded, contains 16,569 base pairs, and encodes thirty-seven genes (Anderson *et al*., 1981). Thirteen of these genes produce proteins involved in oxidative phosphorylation and the remaining genes encode transfer RNAs and ribosomal RNAs necessary for the transcription and translation of the mtDNA genome (Ingman & Gyllensten, 2001). The two strands of mtDNA are referred to

as the heavy strand and the light strand and are differentiated by their nucleotide composition. The heavy strand has more cytosine-guanine residues than the light strand and contains approximately three times the number of genes as the light strand (Bielawski & Gold, 2002). What makes mtDNA unique when compared to nuclear DNA is its inability to recombine, its increased rate of substitution, maternal inheritance, and the ability to isolate it from severely degraded samples (Ingman & Gyllensten, 2001).

The noncoding region of the mtDNA genome, often called the control region or D-loop, is about 1,200 base pairs in length (Hoong & Lek, 2005). This region contains the origin of replication for the heavy strand and controls RNA and DNA synthesis (Chang & Clayton, 1985). It is highly polymorphic and accumulates point mutations at about ten times the rate of nuclear DNA (Hoong & Lek, 2005; Ingman & Gyllensten, 2001). The high level of substitution can be "attributed to a lack of mitochondrial histones, a high concentration of oxidative radicals" and a reduced number of proofreading enzymes (Ingman & Gyllensten, 2001). The D-loop contains hypervariable (HV) regions HV1 and HV2 that are used extensively for forensic analysis due to their high variability (Hoong & Lek, 2005).

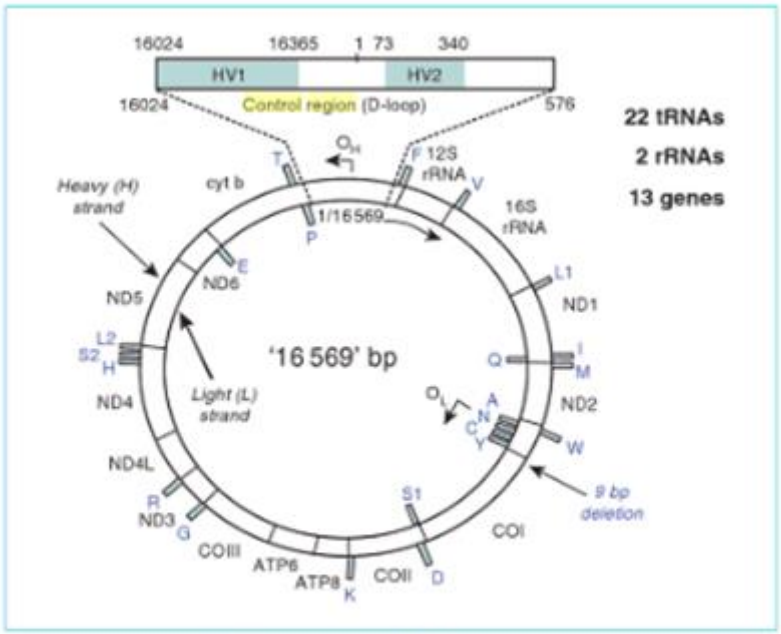


Figure 6: Image of the mitochondrial DNA genome. (Butler, 2005)

The increased rate of mutation of the mitochondrial genome can cause multiple mtDNA types to be present in a single individual, a phenomenon defined as heteroplasmy (Melton, 2004). Heteroplasmic mutations occur preferentially at the hypervariable regions and can be observed as sequence or length mutations (Bendall & Sykes, 1995; Bendall *et al*., 1996; Gryzbowaki *et al*., 2003; Melton, 2004). Sequence heteroplasmy is detected by the "presence of two nucleotides at a single site which show up as overlapping peaks in a sequence electropherogram" (Melton, 2004). Length heteroplasmy is generally observed within the homopolymeric C-stretches of the hypervariable region, specifically in HV1 at positions 16184-16193 and HV2 at positions 303-310 (Stewart *et al*., 2001). Heteroplasmy "segregates differentially in different tissues" (Gryzbowaki *et al*., 2003). The ratio of bases may not be conserved between tissue types or between multiple hairs from the same individual (Gryzbowaki *et al*., 2003; Sullivan *et al*., 1997; Sekiguchi *et al*., 2003). For sequencing of mtDNA from hair, it is recommended that multiple hairs from an individual be analyzed to confirm the presence of heteroplasmy**.**

Human Hair Extensions

Human hair extensions are manufactured from human hair that has been cut and treated with a combination of acids and other chemicals that are often proprietary in nature. The fact that hair extensions are made from cut human hair with no hair follicles intact make them unusable for nuclear DNA characterization (Wilson *et al*., 1995). However, the shaft of the hair contains mitochondrial DNA that can be potentially extracted, quantified, and sequenced (Graffy & Foran, 2005; Linch *et al*., 2001). A limitation to mtDNA is that there is little to no discrimination between individuals of the same maternal lineage, making an identification to the donor impossible (Baker *et al*., 2001). This limitation has minimal relevance to this study as the objective is not to determine the identity of the hair donor, but to identify the hair as an extension.

The chemical treatment and manufacturing processes associated with the production of human hair extensions may cause DNA degradation. However, the circular genomic structure of mtDNA make it less susceptible to exonuclease activity and allow DNA to be recovered from damaged, degraded, or very small biological samples (Hoong & Lek, 2005; Wilson *et al*., 1995). Moreover, most human cells contain thousands of copies of mtDNA genomes increasing the sensitivity of detection and the likelihood of recovering sufficient DNA from compromised samples (Baker *et al*., 2001; Wilson *et al*., 1995).

Although mtDNA is less discriminatory than autosomal DNA analysis, it can be used to associate an unknown sample with a particular population (Cann *et al*., 1987). The Genographic Project, launched by National Geographic in 2006, has identified variations within genetic markers in the mtDNA genome that are intrinsic to specific populations. Data from over 78,000 mtDNA variants from key indigenous populations, including East Asia, India, the Middle East, North America, North Eurasia, and sub-Saharan Africa, have been compiled into a database. The database provides a geographic and cultural context with which to compare unknown samples, resulting in regional affiliations for unknown genomes (National Geographic, 2012; TallBear, 2007). In the analysis of processed human hair extensions, this technology has the capability of determining the country of origin or heritage of the individual who donated the hair. As Asia supplies the majority of the hair to the extension market (Biel, 2010), knowing the country of origin is useful in identifying a hair as an extension.

mtDNA Analysis of Human Hair

DNA extraction uses a combination of physical and chemical techniques to isolate genetic material from a cell. Extraction requires cell lysis facilitated by the addition of a surfactant, removing proteins with a protease, separating the soluble DNA from cell debris, and purifying the

DNA (Promega, 2014). Purification takes advantage of the structure of the DNA molecule and its affinity for particular mediums. For example, in organic extraction, DNA separates into the aqueous phase as the negatively charged DNA is attracted to the polar water (Buckingham, 2012). The aqueous phase is separated from the organic phase and the DNA is precipitated into a pellet. In mini-column purification, DNA is adsorbed to a silica membrane in the presence of high concentrations of chaotropic salt (Boom *et al.,* 1990, Chen &Thomas, 1980). "The binding of DNA to silica seems to be driven by dehydration and hydrogen bond formation" (Melzak *et al.* 1996). Isolation of mtDNA from human hair utilizes one of many possible extraction techniques including phenol/chloroform organic extraction (Wilson *et al.,* 1995), alkaline digestion (Graffy *et al*., 2005), and commercialized extraction kits (Promega, 2012). These methods are described in greater detail in the literature review.

Following extraction, UV spectrophotometry can be used to quantitate and determine the purity of a DNA sample. DNA absorbs light strongly at a wavelength of 260 nm. The amount of light absorbed is directly proportional to the concentration of DNA and, therefore, the concentration of DNA can be quantified using a spectrophotometer according to the Beer-Lambert Law (Clark & Christopher, 2000). The following equation can be used to determine the total concentration of DNA in an unknown sample:

$$[\text{DNA}]\ (\mu g/ml) = OD_{260} \times (\text{dilution factor}) \times 50\ \mu g/ml$$

OD_{260} is the light absorption at 260 nm

The equation assumes that the sample is pure double stranded DNA with an OD_{260} of 1 and concentration of 50 μg/ml. The purity of a DNA sample can be determined by finding the ratio of the absorbance at 260 nm to the absorbance at 280 nm (OD_{260}/OD_{280}) (Clark & Christopher, 2000;

Thermo, 2011). For a pure DNA sample, the value of the ratio should be about 1.8. Elevated ratios usually indicate the presence of RNA and ratios below 1.8 often signal the presence of proteins.

Polymerase chain reaction (PCR) is used for the selective amplification of the control region of extracted mtDNA (Saiki *et al*., 1988; Wilson *et al.*, 1995). PCR is an enzymatic, *in vitro* method that exponentially increases the DNA sequence by a factor of 10^6 (Saiki *et al*., 1988). A PCR reaction consists of the extracted template DNA, a set of synthetic oligonucleotide primers that flank the target sequence, $MgCl_2$, reaction buffer, deoxynucleotide triphosphates (dNTPs), and the thermostable enzyme *Taq* DNA polymerase (Erdem *et al.*, 2011).

Amplification is accomplished by placing the reaction in a thermal cycler and subjecting it to a series of temperature cycles for specific lengths of time. Each cycle of PCR includes steps for template denaturation, primer annealing, and primer extension (Saiki *et al*., 1988). The initial step, denaturing of the target DNA, effectively separates the complementary strands producing the necessary single stranded DNA template for replication by *Taq* DNA polymerase. This occurs at about 94ºC (Erdem *et al*., 2011). During the annealing step, the oligonucleotide primers bind to regions on the template DNA that flank the target sequence (Saiki *et al*., 1988). This identifies the sequence to be amplified and acts as

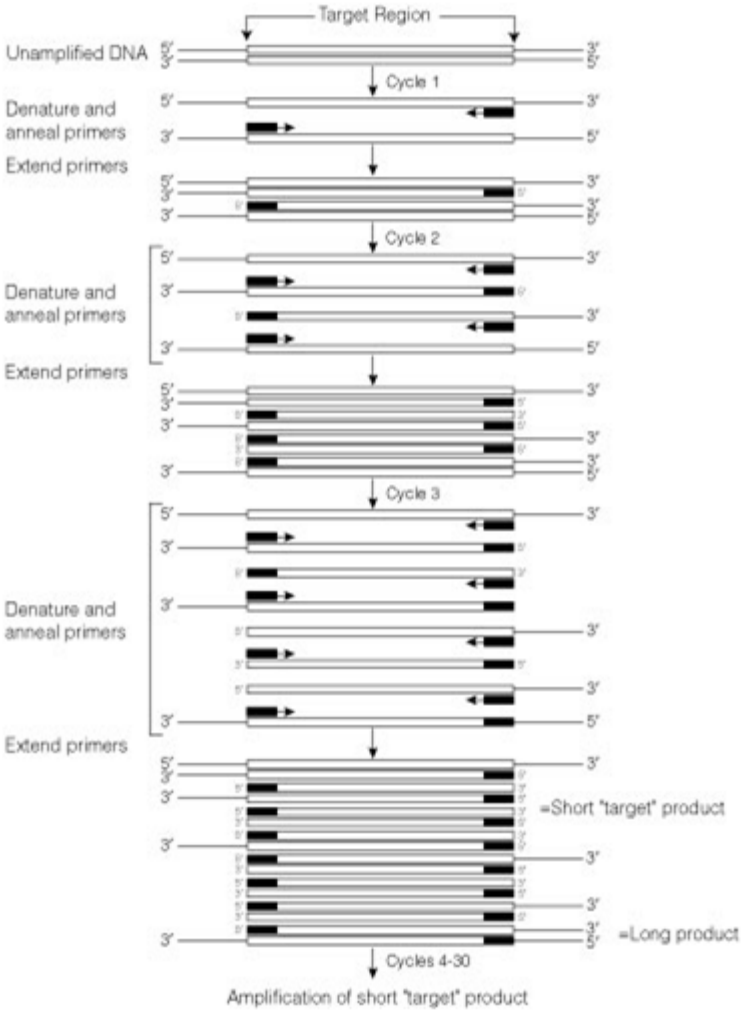


Figure 7: Schematic of the PCR Process (Promega, 2014)

a primer for the DNA dependent DNA polymerase. Annealing temperature is primer specific and for the amplification of HV1 and HV2 of the mtDNA genome approximately 57ºC (Erdem *et al*., 2011). Synthesis is initiated by raising the temperature to 72ºC, which is the optimum reaction temperature for *Taq* DNA polymerase (Erdem *et al*., 2011; Saiki *et al*., 1988). The DNA polymerase adds free dNTPs from the reaction mixture to extend the primer and generate a new strand of DNA. mtDNA PCR amplification requires 32 reaction cycles and generates millions of copies of the DNA sequence.

To increase primer specificity and prevent spurious annealing, touchdown PCR thermal variation can be used for amplification (Don *et al.*, 1991). This technique decreases the primer annealing temperature by 0.5ºC every cycle until reaching a final or touchdown temperature. The annealing temperature range for mtDNA is 50º- 60ºC and amplification requires forty reaction cycles.

Sequencing of mtDNA amplicons generated by PCR is most often accomplished by cycle sequencing. Cycle sequencing employs the Sanger method of DNA sequencing to determine the arrangement of nucleotide bases in a mtDNA sample (Sanger *et al*., 1977). Also referred to as the as the dideoxy-terminator method, the Sanger sequencing technique requires a single stranded DNA template, a single DNA primer, *Taq* DNA polymerase, deoxynucleotidetriphosphates (dNTPs) and dideoxynucleotidetriphosphates (ddNTPs). ddNTPs are modified nucleotides that lack a 3'-OH functional group which is required for the formation of a phosphodiester bond between two nucleotides. ddNTPs function to terminate DNA strand synthesis and are fluorescently labeled for detection in an automated instrument (Sanger *et al*., 1977; Stranneheim & Lundeberg, 2012).

Cycle sequenced DNA is separated by capillary electrophoresis and sequences are interpreted and edited by genetic software. Software capabilities include aligning forward and reverse sequences to generate a consensus sequence, comparing sequences to the revised Cambridge sequence, and identifying single nucleotide polymorphisms (SNPs). SNPs, which have a very low mutation rate and are generally conserved across the genome, can be used to assign haplotypes and predict ethnicity (Allocco *et al.*, 2007)

Microscopic Analysis of Hair

Forensic hair characterization by microscopy is used in the analysis of hair for determining species type, classifying general characteristics, and for the evaluation of unique features (Deedrick and Koch, 2004a). Animal and human hair exhibit different characteristics varying in pigmentation, shape, shaft diameter, root appearance, scale pattern, and internal features (Deedrick and Koch, 2004b; Wheeler & Wilson, 2008). Human head hair is generally consistent in color throughout the length of the hair, has an imbricate or irregular waved scale pattern, a shaft diameter that is generally moderate with gradual changes, and an amorphous medulla which, depending on the hair sample, is fragmented, discontinuous, or continuous. Human head hair varies in shade and intensity and pigment differs in granule size, shape, and density. Pigmentation is generally distributed toward the cuticle in human hair. These characteristics are enhanced by light microscopy (Deedrick and Koch, 2004a). Hair color can be objectively determined by reflective spectrophotometric measurements or digital imaging (Vaughn *et al*, 2009). Human hair characteristics depend largely on hair type, Caucasian, Negroid, or Mongoloid, as outlined in Table 1 (Deedrick and Koch, 2004a).

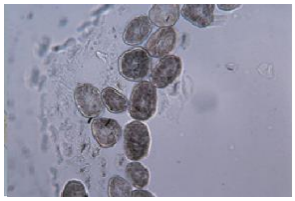
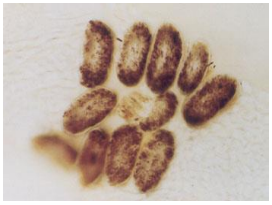
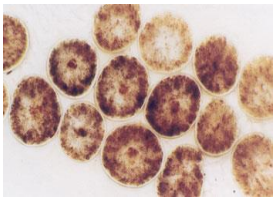
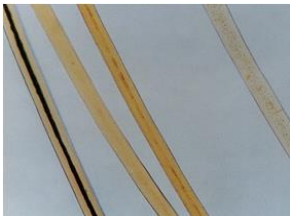
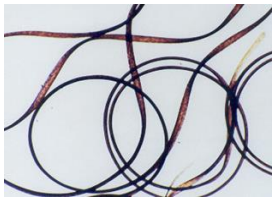
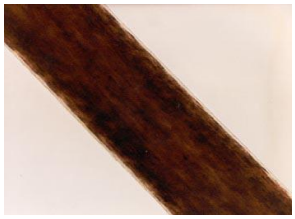
	Caucasian	Negroid	Mongoloid
Shaft Diameter	Moderate with minimal variation	Moderate to fine with considerable variation	Course with little to no variation
Pigment Granules	Sparse to moderately dense with even distribution	Densely distributed and arranged in prominent clumps; hair shaft may appear opaque	Densely distributed and often arranged in large patchy clumps or streaks
Cross-Sectional Shape	Oval 	Flattened 	Round 
Photo-micrograph		Shaft with prominent twist and curl 	Prominent medulla; thick cuticle 

Table 1: Head hair features that indicate racial origin. (Deedrick and Koch, 2004a)

Hair samples with acquired traits from artificial treatment or environmental exposure exhibit distinct characteristics (Deedrick and Koch, 2004a). Hair that has been artificially colored has an "unnatural cast" of color and dye throughout the cuticle and the cortex. Bleaching hair removes pigment, presenting a distinct yellowing of the hair and the development of cortical fusi. Repeated treatment can be identified by multiple "lines of demarcation." Chemical treatment, repeated dyeing, or exposure to harsh environmental conditions may damage the scale structure of the hair or make the hair brittle (Deedrick and Koch, 2004a; Hicks, 1977). Significant damage to hair can also be identified microscopically (Deedrick and Koch, 2004a; Hicks, 1977). Crushed hairs are characterized by a widening of the hair shaft and damaged, separated cortical cells.

Broken hairs tend to have "elongated tags or fragments attached" and burned hairs are charred and fragile exhibiting circular vacuoles at the location of the burn.

Certain diseases or deficiencies can cause banding and significant diameter fluctuations. The phenotypes associated with these disorders can be observed using microscopic techniques. Pili annulati is an autosomal dominant hair condition that is characterized by follicular hyperkeratosis and hairs with alternating dark and light bands (Green *et al*., 2004). The ringed appearance is caused by cavities in the cortex. Monilethrix is another autosomal dominant hair disorder that causes fragile hair with a beaded appearance (Bindurani & Rajiv, 2013). The beading is a result of significant variations in diameter along the hair shaft.

Forensic hair analysis requires a high level of detail and a series of techniques and instrumentation are used for the examination of hair including stereomicroscopy, polarized light microscopy, and scanning electron microscopy. Stereomicroscopes are used to evaluate macroscopic features of hair like color, length, shape and texture. Examination by a stereomicroscope allows for hairs to be differentiated from fibers, assessed for comparison value, somatic region determined, trace materials on the hair to be visualized and identified, and roots evaluated for nuclear DNA analysis (Scientific Working Group on Materials Analysis, 2005). This technique is limited in that it cannot be used for the individualization of the hair as it only determines general characteristics (Wheeler & Wilson, 2008).

Polarized light microscopy uses transmitted light to examine the internal characteristics of hair including color, distribution of pigment granules, presence of ovoid bodies and cortical fusi, shape and structure of the medulla, and cross-sectional shape (Deedrick and Koch, 2004a; Scientific Working Group on Materials Analysis, 2005). Ocular micrometers are used with this technique for measuring the shaft diameter of the hair (Wheeler & Wilson, 2008). Polarized light

microscopy can identify unique characteristics and help determine racial origin (Deedrick and Koch, 2004a; Wheeler & Wilson, 2008). It does not allow for surface characteristics to be examined (Verhoeven, 1972). In forensic analysis, comparison microscopes use polarized light technology, but are specifically designed for the side-by-side comparison of a known hair to a questioned hair (Scientific Working Group on Materials Analysis, 2005).

Scanning electron microscopes (SEM) produce images by scanning samples with a beam of electrons (Wheeler & Wilson, 2008; Zhou *et al*, 2007). For hair samples, SEM technology can provide information on topography and composition (Verhoeven, 1972; Zhou *et al*, 2007). This technique magnifies the surface of a hair allowing for the visualization of scale patterns, damage to the surface, pitting/striations on the hair, and trace material deposited on the surface (Verhoeven, 1972). SEM instrumentation in conjunction with an energy-dispersive detector (EDX) provides elemental analysis to identify the elements present in a sample and relative amounts of these elements (Wheeler & Wilson, 2008; Zhou *et al*, 2007). For hairs, carbon, oxygen, nitrogen, and sulfur are expected elemental components (Bowen, 1979). Metallic elements like aluminum and calcium may be deposited on hair during growth or absorbed from environmental exposure (Hicks, 1977). Although SEM generates high resolution images, it is limited in that it cannot evaluate internal characteristics and only black and white pictures are produced (Wheeler & Wilson, 2008; Zhou *et al*, 2007).

Literature Review

This study examined the forensic significance of processed human hair extensions and attempted to distinguish them from real human head hairs. There is currently a lack of forensic information on hair extensions made from human hair and no published methodology for their evaluation. Without direct references, the literature review and research were conducted using traditional hair studies. Also, multiple mtDNA extraction techniques for hair were evaluated in the literature review to identify the best approach for the analysis of processed human hair extensions.

Analysis of Hair Extensions

A review has identified only a single publication in the literature which references forensic analysis of hair extensions - *Forensic Mitochondrial DNA Analysis of 691 Casework Hairs* (Melton *et al*., 2005). Although hair extensions were not the focus of the study, the authors justified a failed analysis by suggesting that the sample may have been non-human or even synthetic, potentially from human-weave hair. Melton *et al.* proposed using microscopic analysis to determine if the hair was synthetic and recommended isolating and sequencing 12s rRNA mtDNA for species determination. No reference was made to human hair extensions or the possibility of isolating mtDNA from human hair extensions.

Extraction, Amplification, and Sequencing of mtDNA from Human Hair

Wilson *et al*. in 1995 described a protocol that used organic extraction to isolate and sequence mtDNA from human hair shafts. This technique was selective for the control region or D-loop of the human mtDNA genome. In this study, hair shafts were ground using a glass micro-tissue grinder and pre-treated with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Organic chloroform, phenol, and isoamyl alcohol were used in a 25:24:1 ratio to isolate the mtDNA from

the hair samples. The product was purified by filtration and amplified by polymerase chain reaction (PCR). PCR primers were selected to flank the hypervariable regions of the D-loop. Following amplification, the PCR products were purified to remove unincorporated PCR primers. The amplicons were then subjected to Sanger cycle sequencing, separated by capillary electrophoresis, and analyzed using an automated DNA sequencer (Sanger & Coulson, 1977). To validate the methodology, mtDNA sequences obtained from the human hair shafts were compared to mtDNA sequences associated with blood samples from the same individual. Genetic polymorphisms from the hair shaft samples were consistent with those identified in the blood samples.

Prior to 1995, mtDNA had been successfully extracted from human hair shafts in other studies. However, most used hairs with the root still attached increasing the likelihood that the samples contained follicular tissue and, thus, nuclear DNA (Higuchi *et al*., 1988; Hopgood *et al*., 1992). These previous methods involved the chemical and enzymatic digestion of the hair prior to extraction, specifically, dissolving the hair at pH 8 using dithiothreitol (DTT), proteinase K, and 2% sodium dodecylsulfate. Although, this was effective for some hair samples, it was inadequate for use on chemically treated hairs that were resistant to digestion. To overcome this problem, Wilson *et al*. (1995) physically crushed the hair shaft instead of treating it with chemicals resulting in an increased accumulation of PCR products.

The most current Federal Bureau of Investigation (FBI) protocol, revised in 2005, deviates slightly from the protocol discussed by Wilson *et al*. (FBI Laboratory DNA Analysis Unit 2, 2005; Wilson *et al*., 1995). The procedures for pre-washing the hair samples prior to extraction differ slightly, although, they use the same chemical reagents. Also, the revised procedures indicate the

need for quantification before PCR. Both protocols use phenol/chloroform organic extraction, PCR amplification, a Microcon^{TM} 100 concentrator, and kits for cycle sequencing.

Graffy and Foran (2005) discussed a simple alkaline digestion procedure for extracting mtDNA from human hair shafts. This extraction technique used 5 N NaOH, a neutralizing solution of concentrated HCL, and 2 M Tris. Treatment with NaOH directly dissolved the hair by hydrolyzing the protein (keratin) associated with the hair shaft and did not significantly degrade or damage the DNA. mtDNA extraction products were amplified by PCR. Sequences were determined using a DEQ 8000 Genetic Analyzer and aligned using Bioedit Sequence alignment editor. To validate the alkaline digestion technique, the sequences from the hair shafts were compared to those from reference buccal swabs.

Graffy and Foran (2005) also compared the effectiveness of alkaline digestion to the glass grinding/organic extraction procedure. Amplification was successful for ninety percent of the samples extracted by alkaline digestion. However, only seventy-three percent of the samples subjected to glass-grinding/organic extraction were amplifiable. The alkaline digestion procedure exceeded the amplification rate of the glass-grinding technique. Both methods yielded DNA with similar quality. Other advantages of alkaline digestion included an expedited DNA extraction process, taking only 6-7 hours compared to 22-24 for glass grinding, and the use of less expensive materials and chemicals. A reduced number of steps were involved in the alkaline digestion technique decreasing the potential for contamination and limiting sample loss. Pigments were present in the extraction products for both methods especially when analyzing dark hairs. Centrifugation of the alkaline digestion products removed the pigment contaminates. Pigments present in the glass grinding extracts could not be isolated. Melanin pigment has been demonstrated to inhibit PCR reactions through sequence specific binding to DNA limiting the

amount of available template (Opel *et al.*, 2010; Yoshii *et al.*, 1992). This may have been the cause of the higher PCR yield for alkaline digestion.

A protocol for STR typing of ancient DNA extracted from hair shafts of Siberian mummies was established by Amory *et al*. (2007). This methodology included steps for hair lysis, DNA extraction, and STR amplification and was validated on modern samples and forensic samples before it was applied to ancient hairs. Although the aim of the study was to develop an extraction protocol for isolating nuclear DNA from hair shafts, it offered an effective method for hair lysis and the successful extraction of mtDNA from hair. Three centimeter pieces of the hair shaft were selected from 5-7 hairs. These sections were cut into 0.5 cm fragments and placed in a 1.5 mL microcentrifuge tube limiting the total length to 15-21 cm. This reduced the occurrence of melanin inhibition during PCR amplification. Hair samples were digested in 500 µL of lysis buffer containing 0.005 M EDTA, 2% SDS, 0.01 M Tris-HCL (pH 8), 0.3 M sodium acetate, 10 mg/mL dithiothreitol (DTT), 0.5 mg proteinase K, and 0.001 M N-phenacylthiazolium bromide (PTB). PTB functions by "cleaving glucose-protein derived crosslinks" resulting in an increase in the percent yield of extracted DNA (Amory *et al*., 2007). Samples remained in the lysis buffer overnight at 55ºC under vertical rotation. DNA was isolated from the lysed samples using phenol-chloroform extraction. A volume of 500 µL of phenyl/chloroform/isoamyl alcohol was added in a 25/24/1 ratio to each sample and centrifuged for 5 minutes (1000 x *g*). The extraction was performed twice and the sample was purified and concentrated. The extraction procedure resulted in a high recovery of DNA from the hair shafts.

Commercial kits have become increasingly popular for the analysis of mtDNA. These kits have standardized many of the methodologies already discussed and allow for the consistent extraction of mtDNA. Promega's Tissue and Hair Extraction kit when used with DNAIQ™

technology has resulted in the extraction of significantly high yields of mtDNA from human hair shafts. This system requires pretreatment of the hair with high concentrations of proteinase K and dithiothreitol (DTT) to digest the hair, denature proteins, and facilitate lysing of the cells. The extraction process successfully lyses, washes, and isolates DNA into an elution buffer. Isolated DNA is purified with the DNAIQ™ system via a paramagnetic resin which binds charged DNA molecules and essentially eliminates PCR inhibitors (Promega, 2012).

Developmental validation studies for Promega's Tissue and Hair Extraction kits and DNAIQ™ technology were conducted in 2002. In these studies, mtDNA was successfully extracted from human hair shafts using the Promega protocol (Mandrekar *et al*., 2002). The hypervariable regions of the mtDNA genome demonstrated high amplification rates and samples were sequenced with an ABI PRISM 310 Genetic Analyzer. Sharizah *et al*. investigated the use of QIAamp® DNA Micro kits for the extraction of mtDNA from burnt scalp hairs (Sharizah *et al.*, 2003). The mtDNA was amplified using primers selective for the hypervariable regions and sequenced using an automated system. Polymorphisms associated with the burnt scalp hairs matched those of the alleged mother of the victim, validating the use of this type of kit for hair analysis.

Erdem *et al*. (2011) optimized procedures for mtDNA sequencing from samples with trace amounts of DNA. mtDNA was isolated from earrings, toothbrushes, q-tips, swabs, gums, razors, and cigarette butts using QIAamp DNA Micro-Kits. Blood samples were taken from participants to generate reference sequences for comparison. The hypervariable regions HV1 and HV2 of the mtDNA genome were amplified in separate PCR reaction mixtures each with a volume of 25 µL. PCR mixtures contained 0.25-1 ng DNA, 3.75 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 2.5 µL 10 X PCR buffer, 0.2 mM DNTP mix and 10 pmol of each primer. Primers were specifically

designed to target the hypervariable regions. Thermal cycling parameters were selected to optimize amplification and the resulting products were purified by Exo-Sap treatment. Cycle Sequencing was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit and products underwent electrophoretic separation on an ABI Prism 310 Genetic Analyzer. Sequences were aligned and compared to the revised Cambridge sequence using sequencing software. Sequence analysis indicated the same polymorphic sites were detected from known blood samples and items with trace DNA. Every participant exhibited unique HV1 and HV2 regions. Results demonstrated the efficacy of this protocol for recovering and sequencing mtDNA.

Don *et al*. (1991) developed a method, touchdown PCR, to "circumvent spurious priming during gene amplification." As shorter sequences take advantage of the repetitive stochastic effect, misprimed sequences tend to amplify at an increased rate causing them to dominate the product spectrum and obscure the bands associated with the longer targeted amplicons. With each PCR cycle, the amount of selected product decreases. Optimization procedures can be performed to inhibit spurious amplification. If the interactions between the primers and the shorter unexpected sequences are relatively unstable, changing the concentration of Mg^{2+} in the PCR reaction mixture or increasing the annealing temperature may prevent primers from binding at the wrong locations. Optimization procedures can, however, be time consuming. Alternatively, the authors suggest using touchdown PCR thermal variation which can improve the "imbalance between correct and spurious annealing" allowing for the amplification of the targeted sequence (Don *et al*., 1991). This technique utilizes an annealing temperature at or above the suggested temperature and then decreases it by 1ºC every second cycle until reaching a final or touchdown temperature. This is completed over a series of ten cycles and generates the correct target sequence at a rate of 4-fold per degree Celsius. A 5ºC change results in a 4^5-fold advantage for the target sequence over

spurious interactions. As amplification occurs over a range of temperatures, this technique can also be utilized for PCR when the annealing temperature is unknown or amplification is unsuccessful.

Don et al. used this strategy to amplify cDNA sequences coding for leukemia inhibitory factor (LIF). PCR under standard conditions generated small bands 100-200 base pairs in length and not the targeted 1100 base pair product. The touchdown technique was used to prevent spurious annealing and resulted in the amplification of the expected LIF cDNA sequence.

Polarized Light Microscopy of Hair

According to Wheeler and Wilson (2008), the microscopic evaluation of human hair involves determining the macroscopic and microscopic properties associated with the hair root, tip, and shaft. The root is located at the "proximal end of the hair" and the tip is the "most distal end of the hair" (Wheeler & Wilson, 2008). The middle of the hair or hair shaft and is composed of three main sections, the cuticle, the cortex, and the medulla. Initially, a stereomicroscope is used to determine the macroscopic characteristics of the hair sample including color, shaft form, diameter, and length. Further testing can then be performed using a compound light microscope to identify microscopic properties. Microscopic features include reflected color, cuticle scales, cortex characteristics including pigment granules, shape of medulla, and root and tip characteristics. Cross-sectional analysis of hair samples should also be performed to determine an exact diameter of the hair shaft using a calibrated ocular micrometer and to identify the cross-sectional shape of the hair (oval, round, flattened). Cross-sectional shape is important for determining racial characteristics. The authors also recommended using a synthetic, permanent, or semi-permanent mounting medium with a refractive index (RI) in the range of 1.5-1.6. The

average refractive index for human hair is 1.55 and selecting a mounting medium with a similar RI reduces contrast.

Bisbing and Wolner (1984) were able to discriminate between the head hairs of fraternal and identical twins using microscopic analysis. Hair comparisons were based on color, pigmentation, structure, cuticular traits, cross-sectional shape, and acquired traits (treatment, abnormalities, artifacts). By examining the hair samples visually and with a microscope, the authors were able to match the hair samples with the correct individual and never with a twin. Correct associations were independent of whether the twins were identical or fraternal. Some of the identical twins differed only with respect to color, while others differed with respect to several characteristics suggesting that hair develops quite differently under varied environmental conditions.

These studies highlight what characteristics should be examined and documented when evaluating hair morphology and comparing hair samples. These features include shaft form, length, reflected color, pigmentation, scale pattern, cortex and medulla characteristics, acquired traits, and cross sectional determination of diameter and racial origin. Microscopically significant internal characteristics produced by the manufacturing and chemical treatment of human hair extensions may be discernible by traditional microscopy techniques.

Scanning Electron Microscopy of Hair

Riggott and Wyatt (1980) examined hairs from rats using a scanning electron microscope (SEM) to compare the physical characteristics of hairs from the rats' bodies to hairs from their heads. The study examined hairs from 25 female and 25 male rats. Hairs were mounted on aluminum stubs using double sided adhesive tape and were sputter coated with a thin layer of gold. The SEM microscope was able to distinguish differences in the cuticular scale size and identify

variations in the structural features of the hairs. Although the article is not focused on the evaluation of human hair, it discusses hair sample preparation techniques for SEM analysis and hair characteristics emphasized by SEM magnification.

Wei *et al*. (2005) used "nanomechanical characterization of human hair to evaluate the effect of cosmetic products on the hair surface." Hair morphology was studied using scanning electron microscopy. The samples included Caucasian, Asian, and African hair at "virgin, chemo-mechanically damaged, and treated conditions." Indentation experiments were also performed on both the surface and cross-section of the hair samples and the indents were studied using SEM.

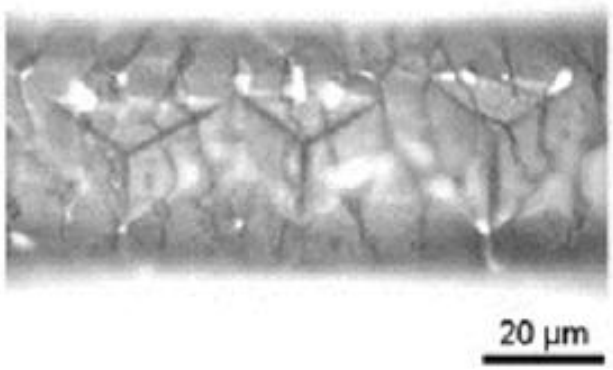


Figure 8: SEM imaging of indentations on human hair. (Wei *et al*., 2005)

The research conducted by Wei *et al.*, specifically the indentation experiments, suggest that SEM imaging will be able to identify striations, pitting, and/or chemical damage on the surface of the processed human hair extensions caused by manufacturing processes.

Microscopy and DNA Analysis

In the article, *Correlation of Microscopic and Mitochondrial DNA Hair Comparisons*, Houck and Budowle (2002) examined the utility of using both microscopy and mtDNA typing in the analysis of hair samples. The authors asserted that neither method alone allowed for positive associations. mtDNA typing could distinguish between hairs of different sources regardless of

morphological features, but could not discriminate between samples from individuals who were maternally linked. This was a distinction that could potentially be made by microscopic analysis. According to Houck *et al*., microscopy provided information on the physical characteristics of the hair (phenotype) and mtDNA typing evaluated the genotype of the source. Ultimately, the authors suggested analyzing all hair samples with a microscope prior to extraction to allow for early exclusions and then exploiting mtDNA technology. This is consistent with recommendations from the Scientific Working Group on Materials Analysis (SWGMAT) (Scientific Working Group on Materials Analysis, 2005).

Techniques from traditional hair studies, including mtDNA sequencing, microscopic evaluations, and chemical analysis can be used to evaluate processed human hair extensions in an effort to differentiate them from real human head hairs. Sequencing of mtDNA can result in the association of haplotypes with the hair extensions.

Methodology

In this study, processed human hair extensions were evaluated to determine their evidentiary value in forensic casework. Comparative analyses between the processed human hair extensions and real human head hairs were performed on an inverted research microscope and scanning electron microscope (SEM) to examine the hair morphology and determine if there were any significant physical or optical differences. Chemical analysis was conducted by the SEM energy dispersive detector (EDX) to identify distinguishing elements present in the hair extensions not found in real human head hairs.

mtDNA was isolated from the extensions using phenol/chloroform organic extraction techniques facilitated by the addition of N-phenylcylthiazolium bromide (PTB) (Amory *et al*., 2007). Isolated mtDNA was quantified using *Nanodrop* UV spectrophotometry (Thermo Fisher Scientific, 2011). Amplification of the mtDNA was accomplished by polymerase chain reaction (PCR) using an Epicentre PCR Failsafe Kit and touchdown PCR thermal variation (Epicentre, 2013; Don *et al*., 1991). mtDNA was sequenced by the Sanger method using the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, 2010). *Sequencher* software was used for sequence interpretation and sequences were compared to the revised Cambridge sequence to determine haplotype.

Processed Human Hair Extensions

Three different grades of processed human hair extensions were evaluated by microscopy and mtDNA analysis. The three brands of extensions used were: Cuticle Remy XQ (Fortified Cuticle High Quality, Caucasian, 100% Human Hair, High Quality), European Caucasian Weave (ECW) (Black and Gold, Caucasian, 100% Human Hair, Medium Quality), and Que by Milky Way (Negroid, 100% Human Hair, Low Quality). High, medium, and low quality designations

were based on subjective criteria assigned by manufacturers. Analysis of the three grades of extensions indicated if each brand had distinguishing microscopic characteristics and if the quality of the extensions affected the isolation of mtDNA

Microscopy

Microscopic examination was used to analyze the characteristics of the processed human hair extensions and verify that they were consistent with real human head hairs. Microscopy also focused on identifying features or damage caused by manufacturing techniques that were unique to the extensions. The hair extensions were evaluated using an Olympus SZ51 Stereomicroscope (Olympus Corporation, Serial Number 4M03682), an MMI Micro-Dissection Inverted Research Microscope (Molecular Machines and Industries, Serial Number 05-10091) and a Hitachi TM3000 Scanning Electron Microscope (Hitachi High-Technologies Corporation, Serial Number 114S2072). Table 2 summarizes the extension samples evaluated by microscopy.

Sample Number	Brand or Donor	Quality/Color
Sample 1	Remy Cuticle Remy XQ – Fortified Cuticle	High/Brown
Sample 2	Remy Cuticle Remy XQ – Fortified Cuticle	High/Brown
Sample 3	Remy Cuticle Remy XQ – Fortified Cuticle	High/Brown
Sample 6	European Caucasian Weaving (ECW) – Black and Gold	Medium/Brown
Sample 7	European Caucasian Weaving (ECW) – Black and Gold	Medium/Brown
Sample 8	European Caucasian Weaving (ECW) – Black and Gold	Medium/Brown
Sample 11	Que by Milky Way	Low/Black
Sample 12	Que by Milky Way	Low/Black
Sample 13	Que by Milky Way	Low/Black
Sample 16	Caucasian – Known	Brown
Sample 17	Negroid – Known	Black
Sample 18	Mongoloid – Known	Black
Sample 19	Caucasian – Dyed Known (semi-permanent black dye)	Black

Table 2: Hair samples analyzed by microscopy.

Stereomicroscopy

The stereomicroscope and the inverted research microscope were used to determine the physical and optical properties of the extensions. As variation can occur within a single hair shaft, the entire length of each hair was examined. The stereomicroscope, which functions at low magnifications (8.0X – 40X, zoom ratio 5:1), was used initially to determine the macroscopic features associated with the surface of the processed human hair extensions. These properties included color, length, and shaft form. Although reflective spectrophotometric measurements are generally used for the objective determination of hair color, the exact color of the hair extensions was not integral to the research and color evaluations were done visually without instrumentation. Color descriptions included black, grey, brown, and light brown. Hairs were examined for variation in color and color intensity along the length of the hair shaft and evaluated for indicators of chemical treatment or dye additives. The length of the hair was measured in centimeters from cut end to distal end. Each sample was measured three times and an average was taken to represent the overall length of the hair. Some of the hair samples exhibited varying shaft forms - convoluting, shouldering, and undulating. Convoluting is described as an abrupt rotation of the hair shaft, shouldering is a partial variation of the diameter along the hair, and undulating features changes in diameter along the entire length of the hair shaft (Wheeler & Wilson, 2008). Analysis was performed according to the SWGMAT guidelines for the evaluation of human hair by stereomicroscopy.

Inverted Research Microscope – Transmitted Light Microscopy

The inverted research microscope was used to analyze internal characteristics of the hair samples using transmitted light at multiple magnifications. Hairs were evaluated using 4X, 10X, and 20X objectives. Combined with the 10X power of the ocular lens, the total magnification was

40X, 100X, and 200X. Hair samples were prepared by mounting them on a glass slide with a cover slip using distilled water as a mounting medium (refractive index = 1.33). Microscopic analysis included the examination of pigmentation color, density, and distribution, presence of scales, cuticle and medulla characteristics, and presence of ovoid bodies and cortical fusi. The approximate width of the cuticle was determined and designated as thick, medium, or thin and if scales could be visualized on the cuticle surface the pattern was described as coronal, spinous, or imbricate. The medulla was labeled as fragmentary, discontinuous, or continuous and the medulla color, shape, and thickness were all documented. The cut end and the distal end of the hair samples were also evaluated. The end shape was described and ends were examined for abnormalities and damage. *MMI CellTools* software allowed for measurement of the shaft diameter. Three different measurements were taken along the length of the hair shaft to account for diameter variation. Images were taken of all hair samples at each magnification. Characteristics were used to determine the possible ethnicity of the hair donor.

Scanning Electron Microscopy

Surface characteristics of the processed human hair extensions were evaluated by a scanning electron microscope (SEM). The SEM uses an electron beam instead of light to form magnified images and provides increased detail and high resolution (Joy, 2009). Four one centimeter cuttings from each hair sample were examined. Samples were mounted on an aluminum stub covered in carbon tape. SEM magnification was adjusted based on the hair sample being analyzed and photographs were taken of all SEM images. SEM analysis highlighted topographical features of the hair samples like flat surfaces, pointed structures, edges, pitting, striations, scales, surface damage, and trace material deposited on the hair. (Dunlap & Adaskaveg, 1997; Verhoeven, 1972). Scale patterns were identified or verified if they had been observed with

transmitted light microscopy. Damage to the hair surface was documented and the possible source of the damage was considered (manufacturing, examiner handling, etc.)

Elemental analysis was conducted using SEM instrumentation and an energy-dispersive detector (EDX). Three EDX tests were conducted at different locations on each 2 cm hair sample. Carbon, oxygen, nitrogen, and sulfur are the expected elemental components of hairs. Metals like aluminum and calcium may also be present. Any trace material or foreign substance observed on the hair surface was photographed and also analyzed by EDX.

Natural human head hairs were examined for comparison purposes. Caucasian, Negroid, and Mongoloid hairs, both virgin and chemically treated (dyed), were microscopically evaluated. Hair samples were collected from female volunteers whose race was self-identified.

Mitochondrial DNA Analysis

mtDNA Phenol/Chloroform Organic Extraction

mtDNA was isolated from two centimeter samples of the processed human hair extensions. Two centimeters is the length designated by the FBI's protocol for mtDNA extraction from human hair (FBI Laboratory DNA Analysis Unit 2, 2005). The hair samples were taken from the hair shaft and were cut into two 1 cm pieces to ensure they were completely covered with solvent during extraction and to reduce the occurrence of melanin inhibition (Amory *et al.*, 2007). Prior to processing, each sample was washed with 1 mL molecular grade water to remove any surface contaminants that may affect DNA isolation or inhibit PCR amplification. Three hairs were processed from each brand of extension for a total of nine extractions. Known hair samples served as positive controls to verify the reliability and consistency of the extraction chemistry. An extraction reagent blank was used to monitor contamination from extraction through sequencing analysis.

mtDNA from the hair samples was isolated and purified using phenol-chloroform organic extraction and ethanol precipitation techniques. Each sample was placed in a 1.5 mL microcentrifuge tube and incubated in "500 µL of lysis buffer overnight at 55ºC under vertical rotation" (Amory *et al.*, 2007). The lysis buffer contained 0.005 M of EDTA, 2% SDS, 0.01 M of Tris-HCl (pH 8), 0.3 M sodium acetate, 10 mg/mL of dithiothreitol (DTT), 0.5 mg proteinase K, and 0.001 M N PTB. PTB effectively cleaves "glucose-protein derived cross-links" aiding in the digestion of the hair samples and increasing the yield of extracted DNA (Amory *et al.*, 2007).

Following digestion, 500 µL of phenol/chloroform/isoamyl alcohol in a 25/24/1 ratio was added to each microcentrifuge tube. The samples were centrifuged at 1000 x *g* for 5 minutes. Centrifugation yielded two distinct phases, the organic phase and the aqueous phase (supernatant). As proteins partition into the organic phase and DNA is retained in the aqueous phase, the supernatant was isolated and transferred to a new 1.5 mL microcentrifuge tube. The product was purified using ethanol precipitation and stored at -20°C until PCR amplification.

DNA Quantification by Ultra-Violet Spectrophotometry

A *Nanodrop* 2000 Spectrophotometer (ThermoScientific, Serial Number 9590) was used to quantify the DNA extracted from the processed human hair extensions. A sample volume of 1 µl of DNA was applied directly to the pedestal of the *Nanodrop,* the arm was closed forming a sample column, and the instrumentation adjusted for an optimal path length. Absorption was determined at 260 nm and 280 nm wavelengths. DNA concentration was measured and a 260/280 ratio was provided to indicate DNA purity. The pedestal surface was wiped clean with ethanol between quantification of different samples.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used for the selective amplification of the control region of the extracted mtDNA. Specifically, 404 base pairs from HVI (nucleotide positions 15997 to 16401) and 374 base pairs from HV2 (nucleotide positions 15 to 389) were amplified for DNA sequencing. These regions were selected because they demonstrate high variability and would be the regions sequenced in a forensic investigation (Takayanagi *et al*., 2003; Wilson *et al*., 1995). Primers flanked hypervariable regions HV1 and HV2 of the D-loop providing a 3' end for DNA polymerase to begin synthesis. Primer pairs were identified as HV1 (15997-F/16401-R) and HV2 (15-F/389-R) (Erderm *et al*., 2011). The sequences associated with these primer sets are summarized in Table 3 and Table 4.

Primer	**Sequence**
15997 – F	CAC CAT TAG CAC CCA AAG CT
16401 – R	TGA TTT CAC GGA TGG TG

Table 3: Primer Sequences Associated with Hypervariable Region 1 (HV1)

Primer	**Sequence**
15 – F	CAC CCT ATT AAC CAC TCA CG
389 – R	CTG GTT AGG CTG GTG TTA GG

Table 4: Primer Sequences Associated with Hypervariable Region 2 (HV2)

PCR amplification was performed using an EpiCentre FailSafe™ PCR Amplification Kit (EpiCentre, #FS99060) and touchdown PCR thermal parameters (Don *et al*., 1991). The FailSafe™ PCR Kit consists of twelve 2X PreMixes. Each PreMix contains a buffered salt solution, all four dNTPs, varying amounts of $MgCl_2$, and FailSafe™ PCR Enhancer (EpiCentre,

2013). The FailSafe™ PCR Enhancer has betaine (trimethyl glycine) which functions to increase "yield and specificity of amplification" and also "protects DNA polymerases from thermal denaturation." Template, primers, and *Taq* DNA polymerase were added to each PreMix and the optimal buffer for amplification was determined by visualization of PCR products on an agarose gel.

A known sample, Sample 18, was used to determine the best PreMix for amplification of the hypervariable regions of mtDNA from hair samples. HV1 reactions consisted of 10 ng mtDNA template, 1.20 µL 15997-F primer, 1.20 µL 16401-R primer, 1.40 µL Ampli*Taq*® DNA polymerase (Life Technologies, #N8080160), 16.35 µL molecular-grade water, and 3.50 µL of one of the FailSafe™ PreMixes (A-L). HV2 reactions had the same components except 15-F and 389-R primers were used. PCR products were run on a 2% agarose gel and Buffer F was identified as the optimal buffer for PCR amplification of both HV1 and HV2. mtDNA isolated from the processed human hair extensions were amplified using the same protocol, reaction mixtures, concentrations, and Buffer F. Each PCR reaction mixture contained a volume of 20 µL.

In an effort to increase sensitivity and primer specificity for the targeted hypervariable regions, touchdown PCR thermal variation was used for amplification of the extracted mtDNA. This technique decreases the primer annealing temperature by 0.5ºC every cycle until reaching a touchdown temperature. This occurs for twenty cycles and amplifies the target sequence at a rate of 4-fold per degree Celsius. Thermal cycling conditions were 95°C for 2 minutes followed by 20 cycles of denaturation at 95°C for 30 seconds, annealing for 1 minute with 0.5°C temperature step-downs every cycle (from 60°C to 50°C), and extension at 72°C for 2 minutes (Don *et al*., 1991). The annealing temperature for the final 20 cycles was 50°C with denaturation and extension phases as above followed by 72˚C for 7 min. The samples were held at 12°C until purification. A PCR

negative control was used to detect the presence of exogenous DNA. Known Sample 18 was used as a positive control to indicate if PCR worked properly.

Following PCR amplification, the samples were purified using MiniElute PCR Purification Kits (Qiagen, #28004). This system uses spin columns for silica-membrane based purification of PCR products (Qiagen, 2008). The purified amplicons were then run on a 2% agarose gel to determine size and verify that the hypervariable regions were amplified. The gel was produced using 4 g agarose, 200 mL of tris-borate/EDTA buffer, and 4 µL of ethidium bromide (EtBr). A total of 5 µL of each sample were added to 2 µL of dye and loaded into the gel. The agarose gel was run at 100 V until the bands were suitably separated. Bands were visualized using a High Performance Ultraviolet Trans-Illuminator (Ultraviolet Products, Serial Number 012405-003) in combination with a Kodak Gel Logic 100 Imaging System and Molecular Imaging Software (Kodak, Serial Number A5-111428-591912-211111).

mtDNA Sequencing

mtDNA from the processed human hair extensions was sequenced using Applied Biosystem's BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, #4337455) and separated by capillary electrophoresis on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Serial Number: 21364-025). Cycle sequencing of both strands of the PCR product used the Sanger sequencing method and was carried out according to the Applied Biosystems' protocol (Applied Biosystems, 2010). Four sequencing reactions were run for each sample: HV1-forward, HV1-reverse, HV2-forward, and HV2-reverse. Cycle sequencing reactions contained 4 µL of ready reaction premix, 2 µL of sequencing buffer, 8 µL of molecular-grade water, 1 µL of the appropriate primer, and 5 µL of amplicon. Sequencing primers were identical to those used in the initial PCR amplification of the extracted mtDNA. Thermal cycling was completed on a

GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Serial Number: 80558201803). The thermal profile included an initial denaturation for 1 minute at 96°C followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The samples were then maintained at 4°C.

After cycle sequencing, the labeled products were purified using EdgeBio Performa® DTR Gel Filtration Cartridges (EdgeBio, #98780) to remove unincorporated dye terminators, salts, and excess primers that interfere with base calling. A volume of 20 µL of each sequencing reaction was added to a packed column and centrifuged for 3 minutes at 850 x *g*. A volume of 10 µl of each eluted sample was combined with 10 µL of Hi-Di™ Formamide (Applied Biosystems, #4311320) in a MicroAmp® Optical 96-well Reaction Plate (Life Technologies, #4316813) and electrophoresed on an ABI PRISM® 3130 Genetic Analyzer.

Sequencing analysis was performed using the genetic software *Sequencher*. An additional HV1 and HV2 sequence, either forward or reverse based on evaluation of peak resolution, was generated for each sample as a means to verify sequences and resolve base call discrepancies. All three sequences were used to generate a consensus sequence. HV1 and HV2 consensus sequences for each sample were aligned and compared to the revised Cambridge Sequence to assign haplotype and identify the ethnicity of the hair extension donor's maternal lineage. Haplotypes were determined based on single nucleotide polymorphisms associated with the mtDNA sequence of each hair extension.

Results

Microscopy

Stereomicroscopy

Macroscopic surface characteristics of the processed human hair extensions and the known human head hairs were evaluated by stereomicroscopy. Results are displayed in Table 5.

Sample	Length	Color	Shaft Form
Sample 1 – Remy XQ	24.87 cm	Brown	Shouldering
Sample 2 – Remy XQ	27.87 cm	Brown	Shouldering
Sample 3 – Remy XQ	30.20 cm	Brown	Shouldering
Sample 6 – ECW	29.10 cm	Brown	Undulating
Sample 7 – ECW	26.90 cm	Brown	Undulating
Sample 8 – ECW	24.60 cm	Brown	Shouldering
Sample 11 – Que	24.73 cm	Black	None
Sample 12 – Que	28.00 cm	Black	Shouldering
Sample 13 – Que	26.07 cm	Black	Shouldering
Sample 16 – Caucasian Known	24.70 cm	Light Brown	Shouldering
Sample 17 – Negroid Known	5.27 cm	Black	Convol.; Undul.
Sample 18 – Mongoloid Known	40.03 cm	Black	Shouldering
Sample 19 – Caucasian Dyed Known (semi-permanent black dye)	32.10 cm	Black/ Dark Brown	Shouldering

Table 5: Stereomicroscope analysis of macroscopic surface characteristics of *Remy XQ*, *ECW*, and *Que* processed human hair extensions and known human hair samples.

The known samples all exhibited characteristics consistent with the ethnicity of the donor. The three hairs analyzed from each brand of extension had similar coloring and length measurements. The color ranged from brown to black. Coloring was comparable to the known human hair samples. The intensity of the black in the *Que* extensions resembled the Caucasian hair treated with semi-permanent black dye indicating the extensions may have been dyed during

manufacturing. Hair extension samples had varying shaft forms indicating changes in diameter along the length of the hair. This was also observed in the known hair samples. Macroscopic features of the *Remy XQ*, *ECW*, and *Que* extensions were consistent with the Caucasian and Mongoloid known human hairs. The extensions did not exhibit features similar to the Negroid known sample.

Inverted Research Microscope – Transmitted Light Microscopy

An *MMI* Inverted Research Microscope was used to analyze the internal characteristics of the processed human hair extensions and the known human head hairs using transmitted light microscopy. Samples were mounted in distilled water, a medium with a refractive index of 1.33. Although a mounting medium with a refractive index between 1.5 and 1.6 would be preferable to visualize the internal features, water was used to prevent chemicals from interacting with the surface chemistry of the hairs and affecting SEM elemental analysis or inhibiting mtDNA extraction and amplification. Examinations were performed at 40X, 100X and 200X magnifications. Hairs were not analyzed at 400X as focusing was inconsistent.

Remy XQ human hair extensions (Samples 1-3) had moderate/medium diameters averaging 82 µm with minimal variation along the length of the hairs. Sample 1 and Sample 2 exhibited tapering near the distal ends. All of the *Remy XQ* hair extensions were brown with changes in intensity along the hair shafts. Sample 2 was lighter in color compared to Samples 1 and 3 and had less color variation. Pigment granules were brown and distributed toward the cuticles. The medullas were opaque, black, discontinuous, amorphous, prominent and broad, and less than one-third the overall shaft diameter. Ovoid bodies were observed in Samples 2 and 3. No cortical fusi were visualized. The cuticles were thick and the outer layer was relatively smooth compared to other extension brands and the known human head hairs. *Remy XQ* claims to have reinforced

cuticles so the smoothness may be attributed to chemical additives or production techniques used during manufacturing. The distal end of each hair extension was rounded.

European Caucasian Weave (ECW) extensions (Samples 6-8) had moderate/medium diameters with distinct variation or undulating hair shafts. The diameters of the extensions averaged 82.67 µm and the hairs were tapered near the distal ends. Sample 6 and Sample 7 were brown with some variation in color and intensity. These extensions exhibited a light/washed out appearance at the distal ends. Sample 8 had a light brown color and no observable changes in color intensity. Pigmentation in Samples 6 and 7 was brown, moderately dense, and distributed toward the cuticle. Pigment granules in sample 8 were brown, sparse, and evenly dispersed. The medullas in Samples 6 and 7 were opaque, black, amorphous, and less than one-third the overall diameter. Darker colored portions of these extension samples had a prominent, broad, continuous medulla, lighter sections had a thin, fragmented medulla, and the medulla was not observable near the distal end of the hairs. The medulla in Sample 8 was visualized along the entire length of the hair shaft. All of the *ECW* extension samples had thick cuticles and no cortical fusi. Ovoid bodies were observed in Sample 6. The distal ends of Samples 7 and 8 were broken and in Sample 6 the distal end appeared to be a dark, opaque bulb although its exact structure could not be characterized by transmitted light microscopy.

Que human hair extensions (Samples 11-13) had moderate to fine diameters that averaged 75 µm. Samples 12 and 13 exhibited diameter variation with the diameter getting smaller near the distal end. The extensions were black/dark gray with minimal change in color along the length of the hairs. The black color presented an unnatural cast and permeated the cuticle and the cortex of the hair shafts indicating treatment with a dye additive. The intensity of the black obscured many of the internal features of the hairs. Pigment granules, medulla characteristics,

and cuticle thickness could not be visualized in the *Que* extension samples. Ovoid bodies and cortical fusi were not observed. The distal ends of the extensions varied in shape. The distal end of Sample 11 was an angled cut, Sample 12 was rounded, and Sample 13 was a square cut with rounded edges.

Microscopic analysis of the processed human hair extensions indicated they were consistent with real human head hairs. In the samples where the medulla could be visualized, the medulla was less than one-third the overall diameter of the hair shaft. This is characteristic of human hairs as animal hairs usually present a medulla that occupies more than half the diameter. Tapering, or a decrease in diameter of the hair near the distal end, was not unique to the extensions. This was also observed in the Caucasian and Mongoloid known samples. Thick cuticles and pigment distributed toward the cuticle of the hair shaft are also intrinsic to human head hairs. Although internal characteristics were not visualized in the *Que* extension samples, analysis of longitudinal sections showed that the extensions were human hair.

The *Remy XQ* human hair extensions and the *European Caucasian Weave* extensions exhibited characteristics consistent with the Mongoloid and Caucasian known samples including diameter, pigment distribution, medulla characteristics, and cuticle characteristics. The black coloring and opaque hair shaft of the *Que* samples inhibited analysis and prevented comparison with known samples. Microscopic examination did not result in definitive ethnic associations for any of the hair extension brands. Ethnicity was ultimately predicted by mtDNA haplotypes.

Manufacturing did not appear to alter the internal characteristics of the processed human hair extensions. Anomalies that were observed were on individual hairs and not repeated on the sample or between samples. Transmitted light microscopy cannot be used to differentiate between a processed human hair extension and a real human head hair.

Sample	Diameter	Color	Pigment	Medulla	Ovoid Bodies	Cortical Fusi	Cuticle	Cut End	Distal End	Other Notes
Sample 1 Remy XQ	Moderate/medium; minimal variation in diameter; tapered near distal end Diameter: 85 µm	Brown: variation in intensity (light to very dark)	Granules: brown; dense; distributed toward the cuticle	Opaque; black; discontinuous; amorphous; prominent and broad; less than one third of overall diameter	Not observed	Not observed	Cuticle: thick Scale pattern: could not be visualized	Blunt square cut; cut by scissors during sample preparation	Rounded	Damage to hair shaft observed
Sample 2 Remy XQ	Moderate/medium; minimal variation in diameter; tapered near distal end Diameter: 79 µm	Light brown: some variation in intensity; hair darker near cut end	Granules: brown; sparse; distributed toward the cuticle	Opaque; black/brown; discontinuous; amorphous; broad; less than one third of overall diameter; not observable along entire length	Observed	Not observed	Cuticle: thick Scale pattern: imbricate; overlapping with narrow margins	Blunt square cut; cut by scissors during sample preparation	Rounded: damaged/crushed	Distal end of hair crushed; crease or indentation observed; scale damage present
Sample 3 Remy XQ	Moderate/medium; minimal variation in diameter Diameter: 82 µm	Brown: variation in intensity (light to very dark)	Granules: brown; distributed toward the cuticle in lighter sections of the hair; could not be visualized in darker sections	Opaque; black; discontinuous; amorphous; prominent and broad; less than one third of overall diameter; not observable along entire length	Observed	Not observed	Cuticle: thick Scale pattern: could not be visualized	Blunt angled cut; cut by scissors during sample preparation	Square cut with rounded edges	Large round dark spots observed
Sample 6 ECW	Moderate/medium; distinct variation in diameter; tapered near distal end Diameter: 83 µm	Brown: variation in intensity (light to very dark); light washed out appearance near distal end	Granules: brown; moderately dense; distributed toward the cuticle throughout most of the hair shaft; evenly distributed in some sections	Opaque; black/brown; amorphous; less than one third of overall diameter; darker portions of the hair had a prominent, broad, continuous medulla; lighter sections had a thin, fragmented medulla; not observable near distal end	Observed	Not observed	Cuticle: thick Scale pattern: could not be visualized	Blunt angled cut; uneven; cut by scissors during sample preparation	Black; opaque; rounded bulb	Distal end – rounded bulb was unique

Table 6: Inverted Research Microscope: transmitted light microscopy of *Remy XQ*, *ECW*, *and Que* processed human hair extensions and known human hair samples.

Sample	Diameter	Color	Pigment	Medulla	Ovoid Bodies	Cortical Fusi	Cuticle	Cut End	Distal End	Other Notes
Sample 7 ECW	Moderate/medium; distinct variation in diameter; tapered near distal end Diameter: 86 µm	Brown: variation in intensity (light to dark); light washed out appearance near distal end	Granules: brown; moderately dense; distributed toward the cuticle throughout most of the hair shaft; evenly distributed in some sections	Opaque; black; amorphous; less than one third of overall diameter; darker portions of the hair had a prominent, broad, continuous medulla; lighter sections had a thin, fragmented medulla; not observable near distal end	Not observed	Not observed	Cuticle: thick Scale pattern: could not be visualized	Blunt square cut; cut by scissors during sample preparation	Broken	None
Sample 8 ECW	Moderate/medium; distinct variation in diameter; tapered near distal end Diameter: 79 µm	Light brown: minimal variation in color and intensity; color darkened when the diameter of the hair shaft decreased	Granules: brown; sparse; evenly distributed throughout most of the hair shaft; distributed toward the cuticle when the diameter of the hair shaft decreased	Opaque; black/brown; discontinuous/fragmented; amorphous; less than one third of overall diameter	Not observed	Not observed	Cuticle: medium thickness Scale pattern: could not be visualized	Blunt angled cut; uneven; cut by scissors during sample preparation	Broken	Section of hair buckled
Sample 11 Que	Moderate/fine; no variation in diameter Diameter: 73 µm	Black/dark grey; opaque; minimal variation in color and intensity; hair shaft may have been dyed	Black color of hair prevented visualization of pigment granules	Opaque; black; discontinuous; amorphous; less than one third of overall diameter Black color prevented visualization of the medulla along most of the length of the hair; medulla in lighter colored sections was observable at high magnifications	Not observed	Not observed	Cuticle: black color of hair prevented thickness from being determined Scale pattern: could not be visualized	Blunt angled cut; uneven; cut by scissors during sample preparation	Angled cut	Black color of hair permeated cuticle and cortex – indicative of dye treatment; may have obscured internal characteristics of the hair

Table 6 continued: Inverted Research Microscope: transmitted light microscopy

Sample	Diameter	Color	Pigment	Medulla	Ovoid Bodies	Cortical Fusi	Cuticle	Cut End	Distal End	Other Notes
Sample 12 Que	Moderate/fine; minimal variation in diameter; tapered near distal end Diameter: 75 µm	Black/dark grey; opaque; no variation in color and intensity; hair shaft may have been dyed	Black color of hair prevented visualization of pigment granules	Black color prevented visualization of the medulla along the entire length of the hair shaft	Not observed	Not observed	Cuticle: black color of hair prevented thickness from being determined Scale pattern: could not be visualized	Blunt angled cut; cut by scissors during sample preparation	Rounded	Black color of hair permeated cuticle and cortex – indicative of dye treatment; may have obscured internal characteristics of the hair
Sample 13 Que	Moderate/fine; minimal variation in diameter Diameter: 77 µm	Black/dark grey; opaque; minimal variation in color and intensity; hair shaft may have been dyed	Black color of hair prevented visualization of pigment granules	Black color prevented visualization of the medulla along the entire length of the hair	Not observed	Not observed	Cuticle: black color of hair prevented thickness from being determined Scale pattern: could not be visualized	Blunt square cut; cut by scissors during sample preparation	Square cut with rounded edges	Black color of hair permeated cuticle and cortex – indicative of dye treatment; may have obscured internal characteristics of the hair
Sample 16 Caucasian Known	Moderate/fine; minimal variation in diameter; tapered near distal end Diameter: 72 µm	Light brown: minimal variation in intensity; slightly darker at root end; exhibited a light washed-out appearance near distal end	Granules: brown; moderately dense; evenly distributed; streaking observed	Brown; discontinuous; amorphous; faint; thin; less than one third overall diameter; not observable along entire length	Observed	Not observed	Cuticle: medium thickness Scale pattern: could not be visualized	Blunt square cut; uneven; small fragment extended passed cut end; cut by scissors during sample preparation	Angled cut	Linear streaking observed in cortex (pigment) Frayed cuticle
Sample 17 Negroid Known	Moderate/fine; considerable variation Diameter: 70 µm	Black; opaque; no variation in color and intensity	Granules: black; dense; evenly distributed; prominent clumping	Opaque; black; discontinuous; amorphous; thin; less than one third of overall diameter Black color prevented visualization of the medulla along most of the length of the hair; medulla in some sections was observable at high mag.	Not observed	Not observed	Cuticle: black color of hair prevented thickness from being determined Scale pattern: could not be visualized	Blunt square cut; uneven; small fragment extended passed cut end; cut by scissors during sample preparation	Frayed; Split-end	None

Table 6 continued: Inverted Research Microscope: transmitted light microscopy

Sample	Diameter	Color	Pigment	Medulla	Ovoid Bodies	Cortical Fusi	Cuticle	Cut End	Distal End	Other Notes
Sample 18 Mongoloid Known	Moderate/course; minimal variation in diameter Diameter: 89 µm	Black/dark grey at the root and root end of the hair; dark brown throughout most of the hair shaft; extreme variation in color and intensity	Granules: black/brown; dense; evenly distributed at the root end of the hair; distributed toward the cuticle throughout the rest of the hair shaft; streaking observed	Opaque; black; discontinuous/ continuous depending on section being observed; amorphous; prominent and broad; less than one third of overall diameter; not observable near the root	Not observed	Not observed	Cuticle: thick Scale pattern: could not be visualized	Root appeared to still be attached (hair was pulled); rounded bulb; dark; opaque; fraying - multiple thin thread-like pieces surrounding root	Blunt angled cut; cut by scissors during sample preparation	Lots of variation observed along length of the hair shaft
Sample 19 Caucasian Dyed Known (semi-permanent black dye)	Moderate/medium; minimal variation in diameter; tapered near distal end Diameter: 80 µm	Light brown/white washed-out appearance at the root and root end of the hair; dark brown, opaque throughout most of the hair shaft; some variation in color and intensity; black dye apparent	Black dye prevented visualization of pigment granules	Light brown, fragmented, thin near the root Opaque; black; discontinuous; amorphous; prominent and broad; less than one third of overall diameter	Observed	Not observed	Cuticle: medium thickness Scale pattern: could not be visualized	Root appeared to still be attached (hair was pulled); rounded bulb; dark; opaque; follicular tag intact	Frayed; Split-end	Semi-Permanent Dye: black streaks densely and evenly distributed; some sections of the hair shaft were more saturated with dye color; still able to visualize internal characteristics of the hair

Table 6 continued: Inverted Research Microscope: transmitted light microscopy

Scanning Electron Microscopy

Scanning electron microscopy was used to analyze the surface characteristics of the processed human hair extensions and the known human head hairs. Four one centimeter cuttings were examined from each sample including the cut end, the distal end, and two cuttings from different locations along the length of the hair shaft. Hair samples were mounted on an aluminum stub covered in carbon tape. SEM magnification varied between 200X and 1000X depending on the hair sample and the features being evaluated. Analysis included examination of surface topography, imaging of the hair sample, and determination of the elemental composition of the hair using EDX technology. SEM images emphasized surface features like scale pattern, damage to the hair shaft, and the presence of trace material on the hair surface. Characteristics unique to the extensions that might be attributed to manufacturing processes or chemical treatment were also identified. **S**EM analysis was limited in that the entire length of the hair was not observed.

Remy XQ human hair extensions (Samples 1-3) exhibited imbricate scale patterns characterized by overlapping scales with narrow margins. The pattern was significantly less pronounced than in the known human head hairs. The *Remy XQ* brand markets a reinforced cuticle suggesting that the lack of definition in the scale pattern may be due to manufacturing processes or chemical treatment of the hair extensions. Significant damage occurred on the surface of the extensions including compressed hair shafts, scale damage, tearing, and lacerations. This is consistent with the damage to the real human head hairs and likely caused by handling of the hair samples. Pitting, striations, and other distinguishing markings were not observed on the *Remy XQ* hair extensions with the exception of wavy striations on Sample 3. These striations were not along the entire length of the hair or observed on other extension samples and therefore not an identifying feature. White, reflective, granules were present on the surface of the hairs. These granules could

potentially be glass from mounting the hairs on glass slides with coverslips during analysis by transmitted light microscopy. This is supported by EDX analysis which identified the white, granules as being composed of silica. Similar granules were observed on almost every other hair extension and on the known hairs. The *Remy XQ* extensions exhibited rounded tips. The known human head hairs all had square or angled cut tips and no other extension brands displayed distal ends with the same unique shape. EDX elemental analysis identified carbon, oxygen, sulfur, aluminum, and calcium in the extensions. Carbon, oxygen, and sulfur are all expected components of hair. Calcium and aluminum are metals that may have been accumulated during growth or acquired from the environment (i.e. hard water, chemicals in beauty products or dyes, etc.). The *Remy XQ* samples had no consistent differences in elemental composition from real human hair.

European Caucasian Weave human hair extensions (Samples 6-8) had imbricate scale patterns with scales that were flattened or compressed. Damage was apparent on all of the *ECW* samples, but was consistent to damage observed on the known human hairs. Pitting and striations were not visualized on Samples 7 and 8. Sample 6 was unique in that the root appeared intact and vertical striations were observed between the root and the hair shaft. The presence of a root indicates that hairs may be randomly attached to the extension thread by either the proximal or distal end during manufacturing and some extensions may be suitable for nuclear DNA analysis. Circular striations on a small portion of the hair shaft were also observed on Sample 6. White, reflective granules were present on the surface of the *ECW* hair extensions and Sample 6 had hair fragments and scales on the surface. The distal ends of Sample 7 and 8 were distinctly split and, although this is observed in real human hair, it may be indicative of *ECW* extensions. EDX analysis indicated carbon, oxygen, sulfur, aluminum, calcium, and silicon in the samples. Calcium was present in the majority of the EDX readings which was elevated from other extension brands.

Silicon may have been from hair products or glass present on the surface of the extensions. *ECW* extensions had no elements that uniquely identified them from real human hair.

Que extension Samples 11 and 13 exhibited imbricate scale patterns. Scales could not be visualized on Sample 12. *Que* samples appeared to have a coating applied to the cuticle which may have obscured the pattern in Sample 12 (F. Springer, personal communication, March 14, 2014). Surface damage was observed on all the samples to include scale damage, tearing, lacerations, and split ends. The damage was not unusual and likely occurred during microscopic analysis of the hairs. No pitting or striations were observed on any of the extensions. White, reflective granules were present on two of the *Que* samples. Carbon, oxygen, sulfur, and aluminum were detected by EDX which is consistent with the composition of human hair.

The processed human hair extensions did not have pitting, striations, or manufacturing indentations consistent between the samples or across brands. *Remy XQ* extensions had less distinguishable scale patterns compared to real human hairs and uniquely rounded tips. *ECW* extensions had compressed scales and split tips. *Que* samples appeared to have a cuticular coating. Although these features may be caused by manufacturing there is no definitive way of determining they are unique to extensions. Accordingly, *Remy XQ*, *ECW*, and *Que* brands cannot be differentiated from real human head hairs based on surface characteristics. EDX analysis indicated that the extensions had the same elemental components as real human hairs.

Sample	Scale Pattern	Substances on the Hair Surface	Possible Manufacturing Characteristics	Elemental Composition	Additional Notes
Sample 1 – Remy XQ	Imbricate Pattern not as pronounced as in real human hair samples Areas where damage occurred pattern was more defined	White, reflective granules observed at the cut end and along the length of the hair shaft	Imbricate scale pattern may be less pronounced than real human hair samples due to chemical treatment or production techniques (reinforced cuticle) Distal end - rounded No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum, Calcium	Distal end – rounded
Sample 2 – Remy XQ	Imbricate Pattern not as pronounced as in real human hair samples Pattern not discernable near the distal end of the hair	White, reflective granules observed at the cut end, distal end, and along the length of the hair shaft	Imbricate scale pattern may be less pronounced than real human hair samples due to chemical treatment or production techniques (reinforced cuticle) Distal end – flat cut with rounded edges No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum	Distal end – flat cut with rounded edges
Sample 3 – Remy XQ	Imbricate Scale pattern not observed in sections with severe damage	White, reflective granules observed along the length of the hair shaft	Distal end - rounded Wavy striations	Carbon, Oxygen, Sulfur, Aluminum	Distal end – rounded Wavy striations not observed along entire hair shaft or on other *Remy XQ* samples

Table 7: Scanning Electron Microscope analysis of hair topography and elemental composition of *Remy XQ*, *ECW*, and *Que* processed human hair extensions and known human hair samples.

Sample	Scale Pattern	Substances on the Hair Surface	Possible Manufacturing Characteristics	Elemental Composition	Additional Notes
Sample 6 – ECW	Imbricate Pattern not as pronounced as in real human hair samples Scales appeared to be flattened or pressed	White, reflective granules observed at the distal end and along the length of the hair shaft Fragments of hair/ sheared scales	Scales were flattened or pressed possibly from a manufacturing technique Root appeared to be intact suggesting hair may be randomly attached to extension thread Vertical striations observed between the root and the hair shaft Circular striations	Carbon, Oxygen, Sulfur, Aluminum	Sample appeared to have a root (rounded bulbous end); root extremely textured with no distinguishable scale pattern Vertical and circular striations not observed along entire hair shaft or on other *ECW* samples
Sample 7 – ECW	Imbricate Scales appeared to be flattened or pressed	White, reflective granules observed along the length of the hair shaft	Distal end of the hair shaft broken/split No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum, Calcium, Silicon	
Sample 8 – ECW	Imbricate Scales appeared to be flattened or pressed	White, reflective granules observed along the length of the hair shaft	Scales appeared to be flattened or pressed possibly from a manufacturing technique Distal end of the hair shaft broken/split No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Calcium	

Table 7 continued: Scanning Electron Microscope analysis of hair topography and elemental composition.

Sample	Scale Pattern	Substances on the Hair Surface	Possible Manufacturing Characteristics	Elemental Composition	Additional Notes
Sample 11 – Que	Imbricate	White, reflective granules observed along the length of the hair shaft Small, white, rectangular object – unidentified	Not observed No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum	
Sample 12 – Que	No distinguishable pattern; hair stripped of scales or possible coating	White, reflective granules observed along the length of the hair shaft	Absence of scale pattern (stripped scales/coating) may have been due to manufacturing processes Distal end – rounded No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum	
Sample 13 – Que	Imbricate	Not observed	Not observed No pitting, striations, or other distinguishing markings	Carbon, Oxygen, Sulfur, Aluminum	
Sample 16 – Caucasian Known	Imbricate	Not observed	N/A – real human head hair	Carbon, Oxygen, Sulfur, Aluminum	
Sample 17 – Negroid Known	Imbricate	Fragments of hair/ sheared scales near the cut end	N/A – real human head hair	Carbon, Oxygen, Sulfur, Aluminum, Silicon	
Sample 18 – Mongoloid Known	Imbricate Pattern less discernable near the root end of the hair - larger scales not as delicately overlaid	White, reflective granules observed at single section of the hair shaft	N/A – real human head hair	Carbon, Oxygen, Sulfur, Aluminum, Calcium	Extreme shearing of scales near the root Root end appeared pulled - extremely textured with multiple threadlike pieces extending from the hair shaft

Table 7 continued: Scanning Electron Microscope analysis of hair topography and elemental composition.

Sample	Scale Pattern	Substances on the Hair Surface	Possible Manufacturing Characteristics	Elemental Composition	Additional Notes
Sample 19 – Caucasian Dyed Known (semi-permanent black dye)	Imbricate; pattern less discernable near the root end of the hair – larger scales not as delicately overlaid Scale pattern not observed at the distal end	White, reflective granules observed at the root end and the distal end of the hair	N/A – real human head hair	Carbon, Oxygen, Sulfur, Aluminum	Distal end severely damaged; split end/broken tip Rounded bulbous root; extremely textured Root had three discernable sections

Table 7 continued: Scanning Electron Microscope analysis of hair topography and elemental composition.

Verification of Microscopy Results

The processed human hair extensions and the known human head hair samples were analyzed at the Sacramento County Laboratory of Forensic Services to verify microscopy results (F. Springer, personal communication, March 14, 2014). According to the lab report, the *Remy XQ* extensions, *European Caucasian Weave* extensions, and all of the known human hairs (Samples 1, 2, 3, 6, 7, 8, 16, 17, 18, and 19) were consistent with human hair in scale pattern and microscopic features. The *Que* extensions, Samples 11, 12 and 13, were dyed black and nearly opaque when analyzed by transmitted light microscopy. Scales on the *Que* samples were damaged or obscured by a coating and were ultimately visualized with reflected light microscopy. Longitudinal sections of the *Que* extensions were taken to see the medulla and pigmentation. These characteristics were consistent with human hair. Identifying features were not observed on the extension samples.

Mitochondrial DNA Analysis

Hypervariable region 2 (HV2) of the extracted mtDNA from the processed human hair extensions was amplified using touchdown PCR thermal parameters, sequenced by the Sanger method, and compared to the revised Cambridge sequence (rCRS) to identify genetic polymorphisms. Analysis of PCR products by agarose gel electrophoresis indicated that hypervariable region 1 (HV1) was not amplified for any of the samples. Re-amplification of HV1 did not generate PCR products and analysis of HV1 was discontinued. Although conclusive haplotype assignments require HV1 and HV2 SNPs, haplotypes can be estimated using just HV2 sequences.

Known Samples 16, 17, and 18 were all extracted and quantified. Known Sample 18 was used as a positive control. Successful amplification and sequencing of Sample 18 indicated that the methodology and reagent chemistry were reliable and appropriate for the analysis of HV2 of the mtDNA genome. An extraction reagent blank was used as a negative control for amplification through sequencing. Exogenous DNA was not detected in the extraction blank indicating reagents were not contaminated.

DNA Quantification

Extracted DNA from the processed human hair extensions and the known human hairs was quantified using UV spectrophotometry to verify there was adequate concentrations of genetic material for subsequent analysis. PCR requires 10 ng of DNA in a 25 µL volume for amplification. Quantification indicated that DNA extracted from the extension samples and the known samples was concentrated enough for PCR. DNA concentration values are displayed in Table 8.

Sample	Measurement 1 (ng/µL)	Measurement 2 (ng/µL)	Measurement 3 (ng/µL)	Average (ng/µL)
Sample 1 – Remy XQ	10.2	9.8	10.0	10
Sample 2 – Remy XQ	12.1	12.2	12.6	12.3
Sample 3 – Remy XQ	30.0	29.6	29.5	29.7
Sample 6 – ECW	20.5	20.8	20.2	20.5
Sample 7 – ECW	43.1	43.8	43.6	43.5
Sample 8 – ECW	18.4	18.5	18.3	18.4
Sample 11 – Que	2.0	2.3	2.0	2.1
Sample 12 – Que	28.1	28.6	28.2	28.3
Sample 13 – Que	41.9	41.8	42.0	41.9
Sample 16 – Caucasian Known	7.0	7.4	6.9	7.1
Sample 17 – Negroid Known	4.1	4.4	4.4	4.3
Sample 18 – Mongoloid Known	18.0	18.6	18.3	18.3

Table 8: Quantification Using *Nanodrop* UV Spectrophotometry - concentration of DNA from the processed human hair extensions and the known human hair samples.

mtDNA Sequencing and Haplotype Assignment

Amplified mtDNA from the *Remy XQ*, *European Caucasian Weave*, and *Que* human hair extensions was sequenced, aligned, and compared to the HV2 region of the revised Cambridge sequence. Sequences for *ECW* Sample 6 and *Que* Sample 12 were not obtained. Analysis of Sample 6 and Sample 12 was not imperative as other hair extensions from the same brands were successfully sequenced. Full sequences are available in Appendix B.

Comparison of each sample's sequence to the rCRS allowed for single nucleotide polymorphisms to be identified and haplotypes predicted. Genetic polymorphisms were designated by the nucleotide position within the mtDNA genome followed by the letter of the mutation. Each sample had a unique combination of SNPs indicating that all of the hair extensions, even those within the same brand, came from different donors. Nucleotide substitutions, deletions, and insertions were observed in the sequences of the extension samples. Deletions were indicated

by ":" symbol and insertions were identified by ".1" following the nucleotide position. Insertions are generally observed in C-stretches and caused by strand slippage of the DNA polymerase. Some of the SNP designations were observed in multiple samples and across brands of extensions (i.e. 73-G, 150-T, 152-C, and 263-G). These SNPs are likely conserved within the mtDNA genome and associated with a particular haplotype. Base differences between the rCRS and the hair extension sequences were contributed to genetic polymorphisms and not heteroplasmy based on peak resolution. Well-defined, single peaks were observed at each position. Heteroplasmy would present multiple peaks at a single location.

Sample	Single Nucleotide Polymorphisms	Possible Haplotypes	Regional Affiliation
Sample 1 – Remy XQ	73-G, 101.1-G, 106.1-T, 150-T, 152-C, 263-G, 309.1-C, 315.1-C	L3 U3	L3: West Central Africa U3: Caucasus and Western Asia
Sample 2 – Remy XQ	73-G, 77-deletion, 150-T, 152-C, 263-G, 309.1-C, 315.1-C, 324-G	L3 U3	L3: West Central Africa U3: Caucasus and Western Asia
Sample 3 – Remy XQ	73-G, 107.1-T, 150-T, 152-C, 263-G, 309.1-C, 315.1-C, 329.1-G, 330-G	L3 U3	L3: West Central Africa U3: Caucasus and Western Asia
Sample 6 – ECW	X	X	X
Sample 7 – ECW	150-T, 152-C, 263-G, 309.1-C, 315.1-C, 324-G	L3 U3 U5	L3: West Central Africa U3: Caucasus and Western Asia U5: Europe
Sample 8 – ECW	73-G, 77-deletion, 185-A, 263-G, 295-T, 304.1-C, 315.1-C, 324-T, 329.1-G	J1	J1: Western Asia, Europe, Caucasus, and North Africa
Sample 11 – Que	X	X	X
Sample 12 – Que	102.1-A, 107.1-T, 107.2-T, 150-T, 152-C, 263-G, 309.1-C, 315.1-C, 329.1-G	L3 U3	L3: West Central Africa U3: Caucasus and Western Asia
Sample 13 – Que	73-G, 94.1-G, 150-T, 152-C, 263-G	U3	U3: Caucasus and Western Asia

Table 9: Haplotypes and regional affiliations of the processed human hair extensions.

Following alignment and SNP identification, the sequence of each sample was subjected to a NCBI DNA BLAST search. All DNA samples from the processed human hair extensions

matched the HV2 region of the mtDNA genome with at least a 98% confidence level. A few samples were also labeled as variants of a nuclear pseudogene. Haplotypes of the sequences that were a near match (> 98%) to the sample sequence being analyzed were recorded. HV2 SNPs allowed for the approximation of haplotypes. Definitive haplotype assignments were not possible without HV1 sequences.

The majority of the processed human hair extensions had L3 and U3 haplotype assignments with regional affiliations to the Caucasus, Western Asia, and West Central Africa. These samples included *Remy XQ* Samples 1, 2, and 3, *ECW* Sample 7, and *Que* Samples 12 and 13. *ECW* Sample 7 also had a U5 haplotype which is associated with European heritage. The only extension sample with a completely unique haplotype when compared to other samples was *ECW* Sample 8. Sample 8 had a J1 designation which is intrinsic to populations from Western Asia, Europe, the Caucasus, and North Africa. Haplotype assignments for the hair extensions represented multiple geographic regions and a huge portion of the population. HV2 genetic polymorphisms were not restrictive enough to determine regional affiliations for particular extension brands or processed human hair extensions as a whole. Sequencing of HV1 is imperative to make haplotype designations.

Initially it was thought that haplotypes would be associated with East Asia (China) and Central Asia (India) as these regions were determined to be the largest contributors of hair to the extension market. However, haplotype approximations based on HV2 SNPs contradict this assumption with regional affiliations to the Caucasus, Western Asia, and North Africa. It was also assumed that a single donor contributed hair to each extension package. mtDNA sequencing of the hair extensions identified hairs from multiple donors in each package. This has significant implications for forensic analysis.

Conclusion

A processed human hair extension recovered from a crime scene would be identified as a shed telogen human hair or a cut head hair. Microscopic evaluation would characterize the extension as having features consistent with human hair and would not be able to definitively classify the hair as an extension. Sequencing of the hypervariable regions of the mtDNA genome would associate the hair donor's maternal lineage with the extension and would not implicate the suspect. Hair extension evidence would be misrepresentative. This has significant implications in forensic case work as a processed human hair extension would not be identified as an extension or excluded as identifying evidence and would misdirect an investigation.

Haplotypes can be assigned from single nucleotide polymorphisms in the mtDNA genome allowing for the association of a hair extension with a particular geographic region. HV2 SNPs provided multiple haplotype possibilities for the processed human hair extensions. The majority of the extensions evaluated were from donors of Caucasus, Western Asian, and West Central African heritage. This represents a large geographic area and multiple ethnicities preventing a direct correlation between processed human hair extensions and specific populations. More definitive haplotype assignments would be possible with HV1 SNP discrimination. Even with HV1 SNPs, haplotype assignments are not an absolute method of hair extension identification as the actual perpetrator of the crime may have the same haplotype. mtDNA haplotypes were consistent with microscopy results which identified most of the hair extensions as having characteristics of Caucasian or Mongoloid (Asian) hair.

mtDNA analysis also identified variations in sequences of extensions of the same brand. This suggests processed human hair extensions are manufactured using hair from multiple donors and that an extension collected from a crime scene cannot be associated to others from the same

manufacturing package by mtDNA sequencing. This would be significant for comparing crime scene samples to extensions still attached to the suspect's head.

Future Research

Chemical assays conducted to identify compounds that are specific to processed human hair extensions could potentially link the extensions back to individual manufacturers. Organic extraction with selected polar and nonpolar solvents might isolate the manufacturing chemicals and allow for the identification of the chemical components by gas-chromatography mass-spectrometry (GC-MS). As the exact chemicals used in the production of processed human hair extensions are proprietary, a series of extractions with different organic solvents would need to be done to account for variability in the molecular weights and functional groups of the compounds being isolated. The extraction process would require sonication to provide specific activation and the extraction products would be reduced with dry nitrogen.

Following organic extraction, the samples would be analyzed by GC-MS. The purified samples would be volatized and separated on a capillary column based on their volatilization temperature and their unique chemical and physical properties (McNair & Miller, 2009). The retention time on the column would be compared to known standards to evaluate the components or analytes of the sample. Additionally, the concentration of the various chemical components would be determined by the area under the curve of the peaks that elute from the GC. The molecular components would then individually be analyzed by a mass spectrometer (MS). The MS determines the elemental composition of each analyte and helps in determining molecular connectivity. These spectra would be compared to reference spectra for molecular identification. Ideally there would be chemicals common among all three brands of processed human hair extensions that are not readily found in real human head hairs. Identifying compounds may include oils, alcohols, hydrocarbons, fatty acids, or other processing chemicals.

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Appendix A

Transmitted Light Microscopy and Scanning Electron Microscopy Images of Processed Human Hair Extensions and Known Human Hairs

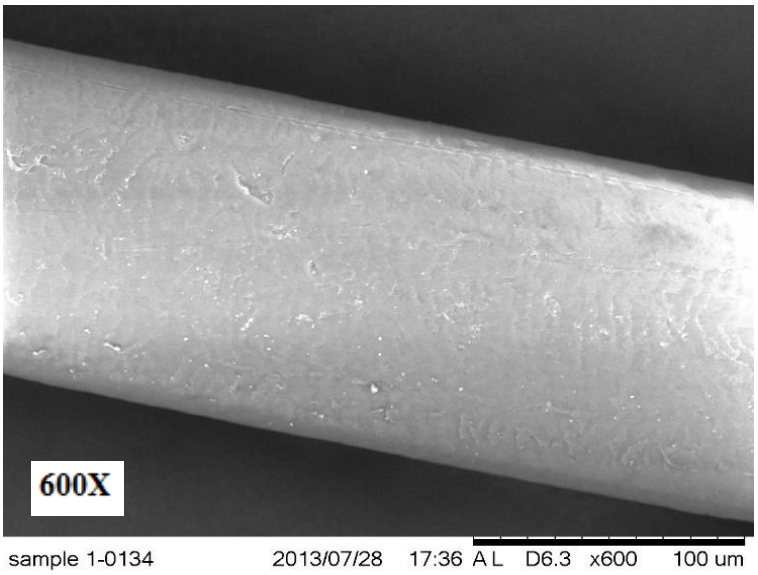
SAMPLE 1 – Remy XQ
Transmitted Light Microscopy

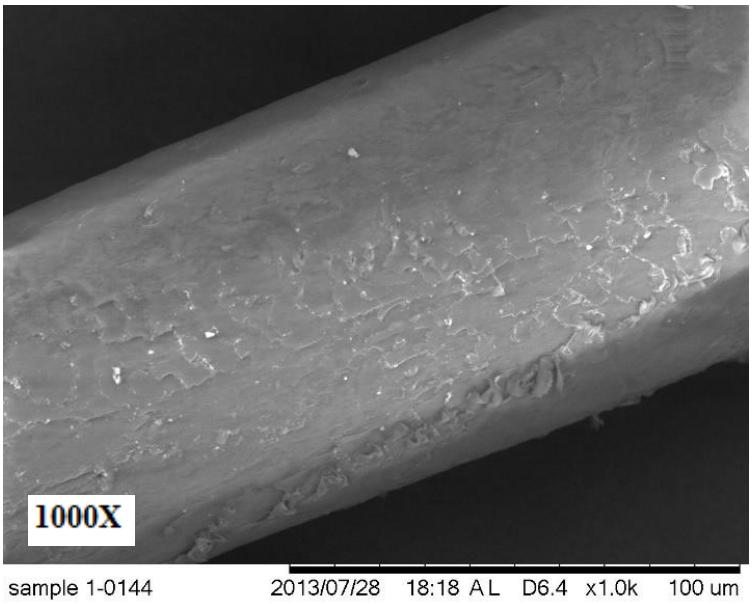

10X

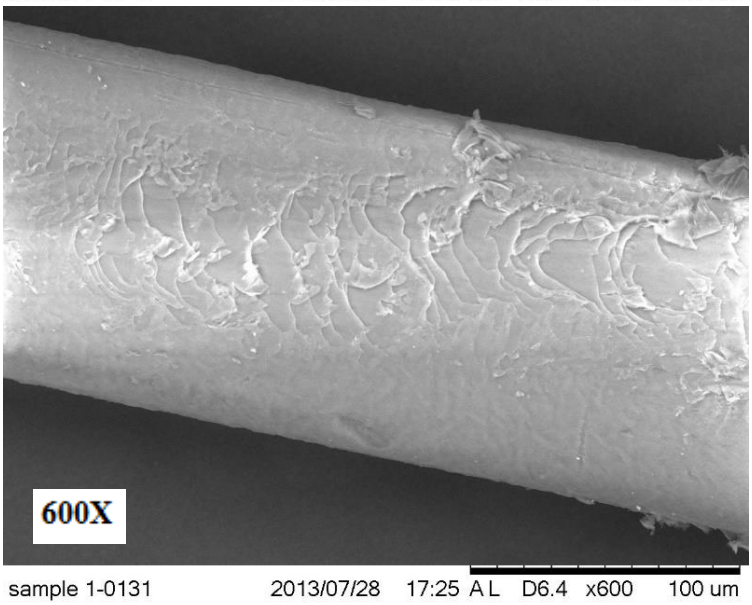

20X

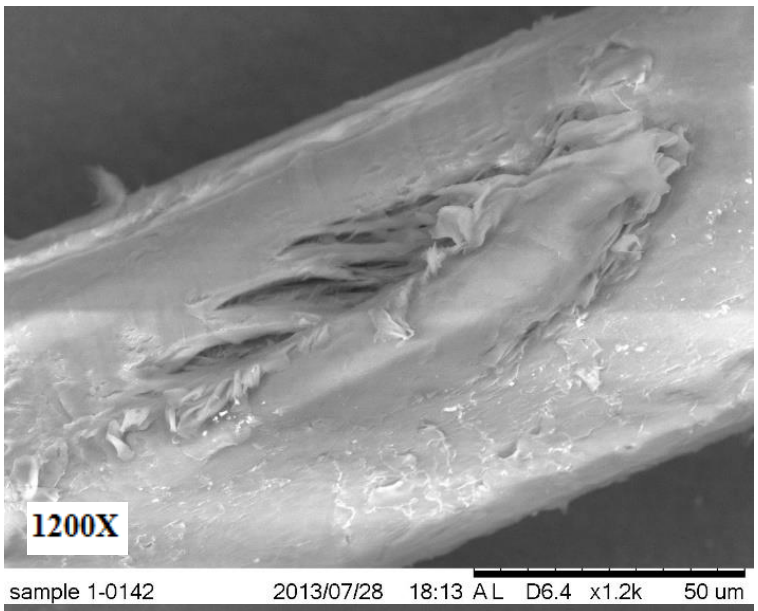

20X

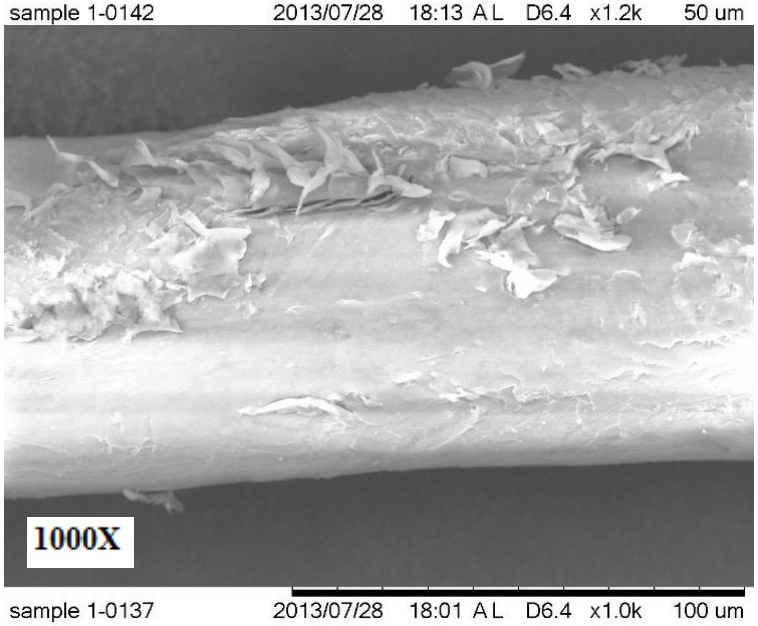
SAMPLE 1 – Remy XQ
Scanning Electron Microscopy







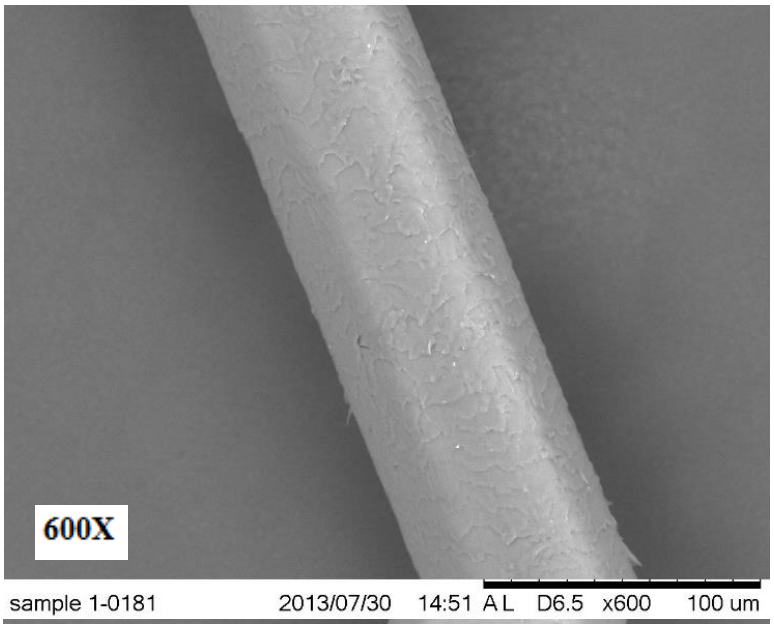

1200X
sample 1-0142 2013/07/28 18:13 A L D6.4 x1.2k 50 um

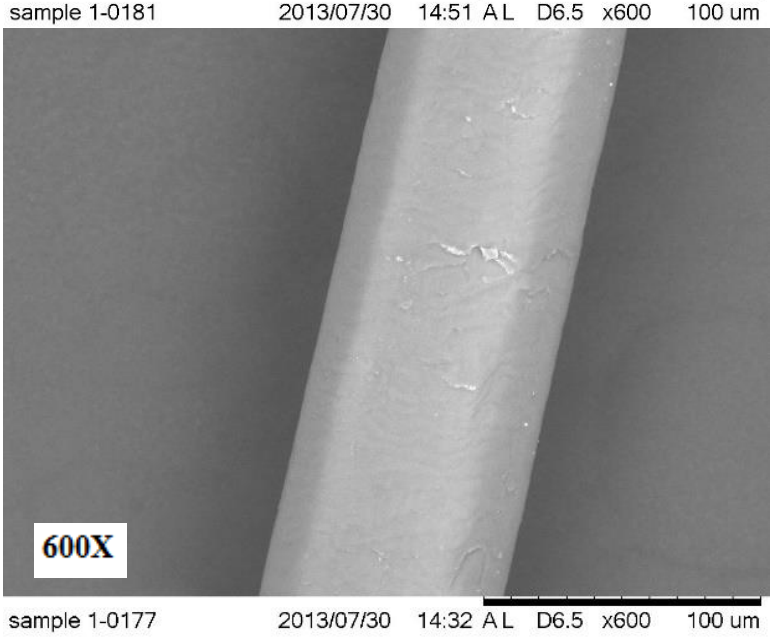

1000X
sample 1-0137 2013/07/28 18:01 A L D6.4 x1.0k 100 um

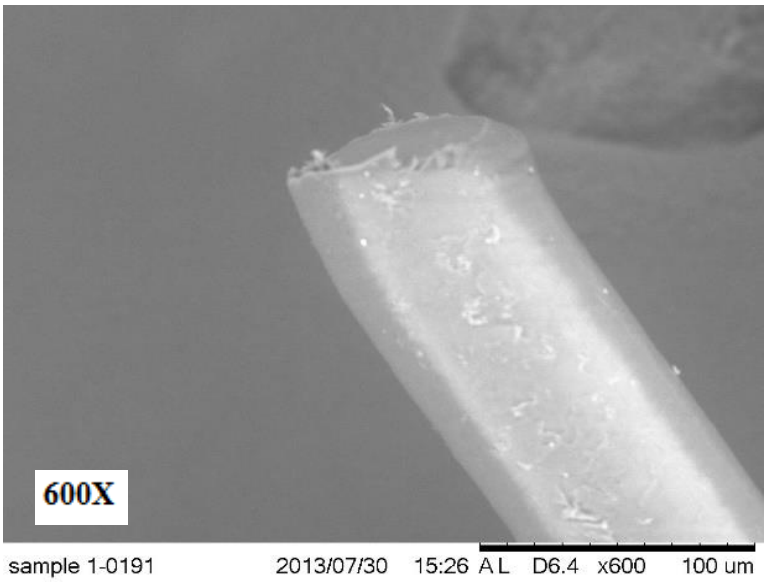

600X
sample 1-0150 2013/07/28 18:55 A L D4.6 x600 100 um

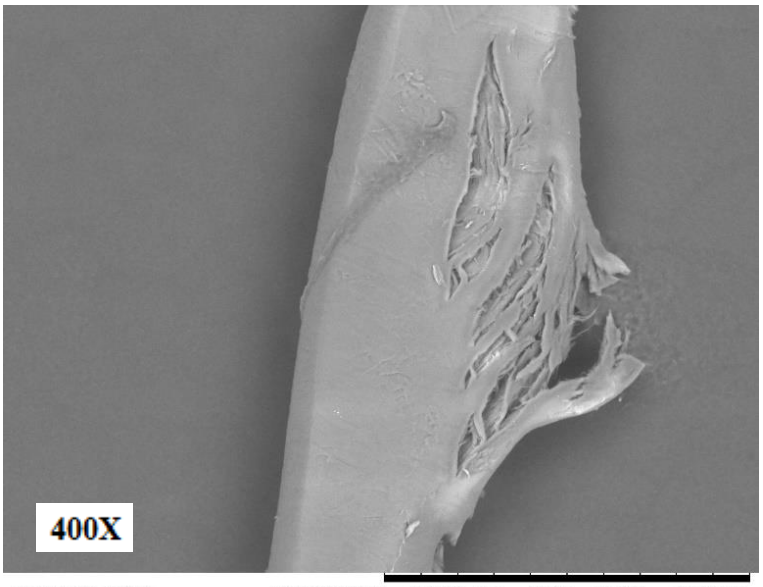
SAMPLE 2 – Remy XQ
Transmitted Light Microscopy

SAMPLE 2 – Remy XQ
Scanning Electron Microscopy

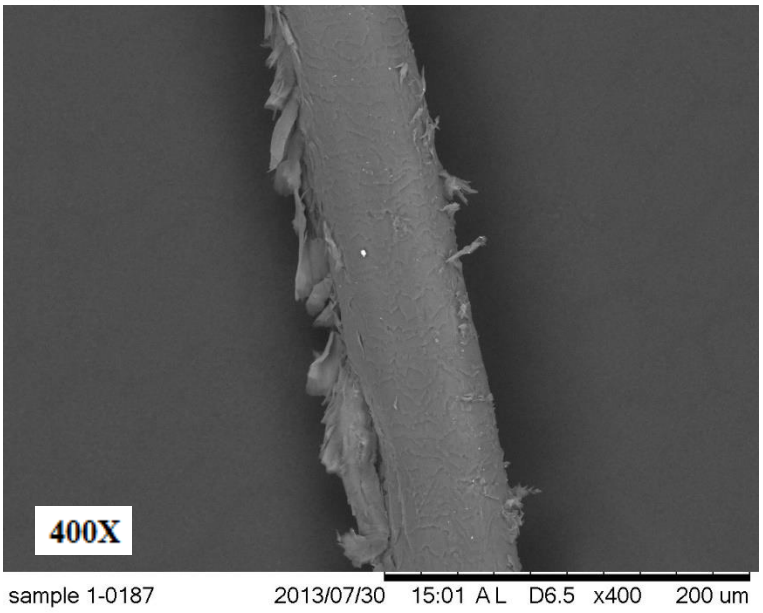






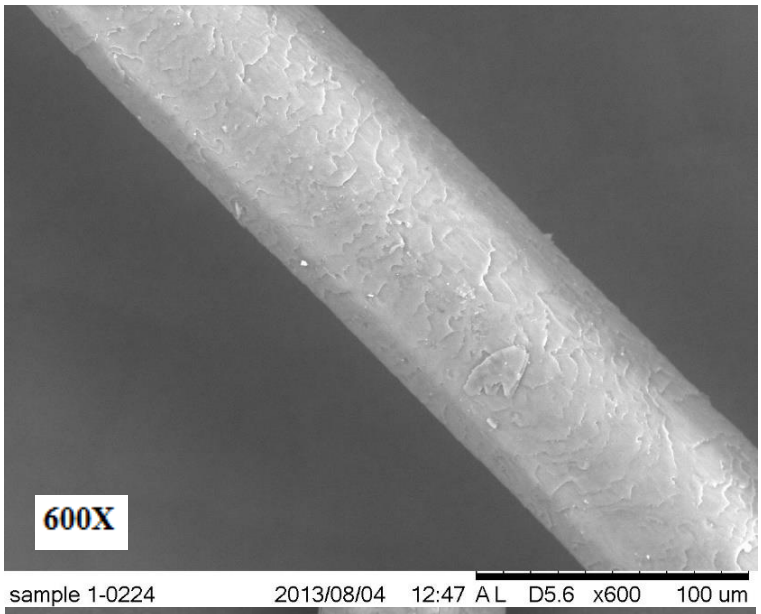

400X
sample 1-0171 2013/07/30 14:18 A L D6.5 x400 200 um

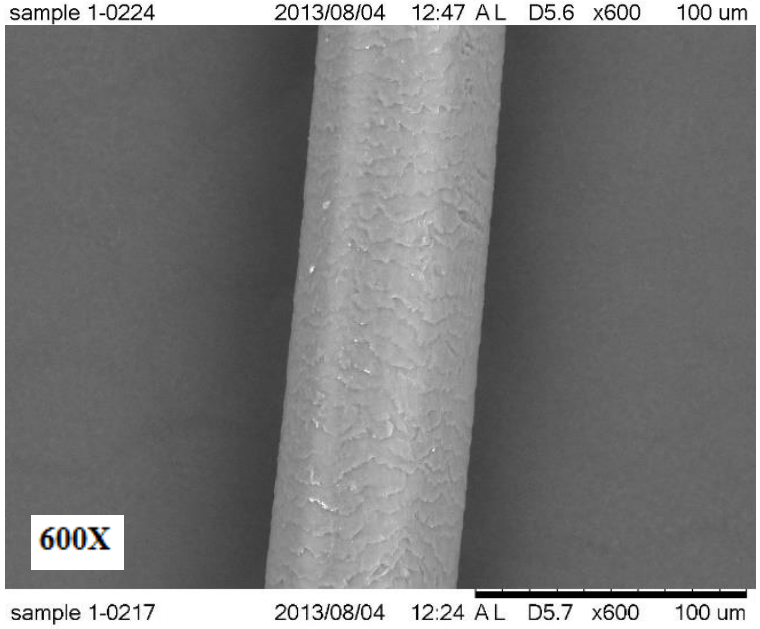

1200X
sample 1-0172 2013/07/30 14:21 A L D6.6 x1.2k 50 um

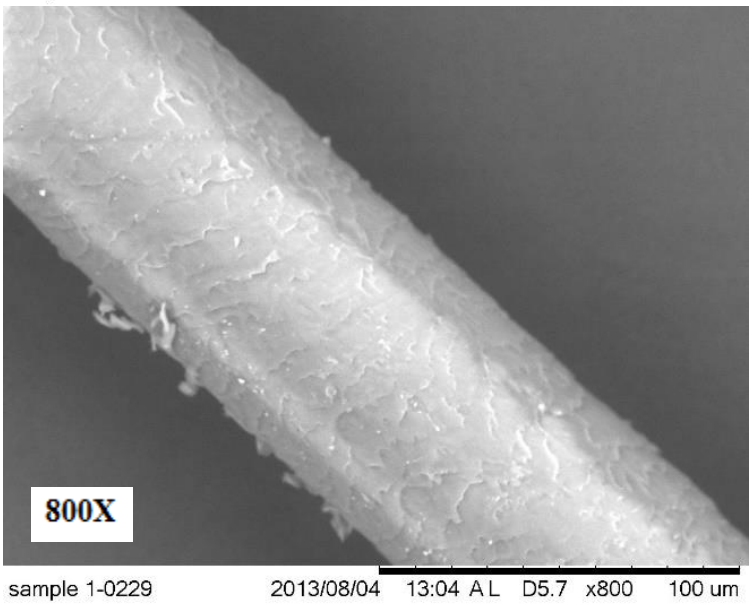

400X
sample 1-0187 2013/07/30 15:01 A L D6.5 x400 200 um

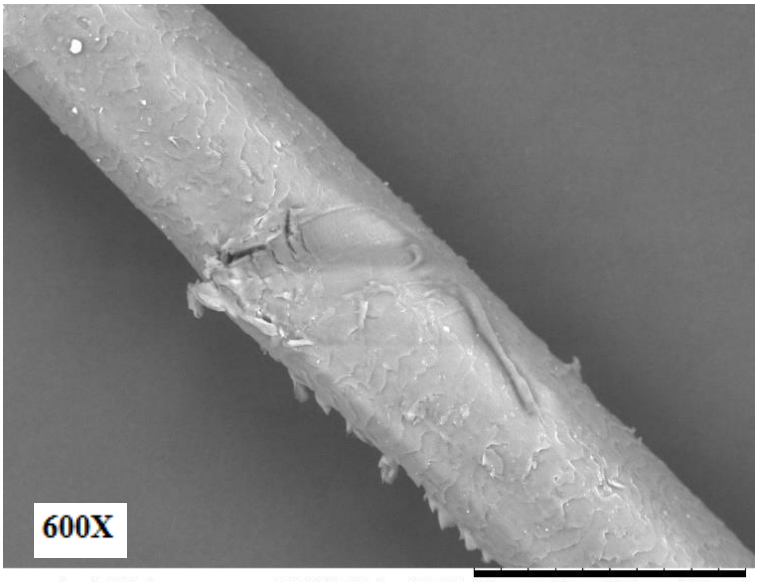
SAMPLE 3 – Remy XQ
Transmitted Light Microscopy

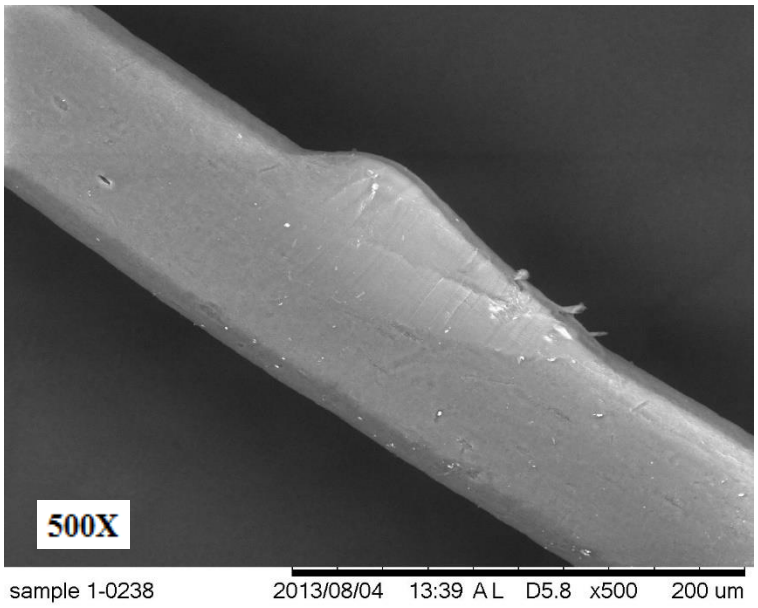
SAMPLE 3 – Remy XQ
Scanning Electron Microscopy

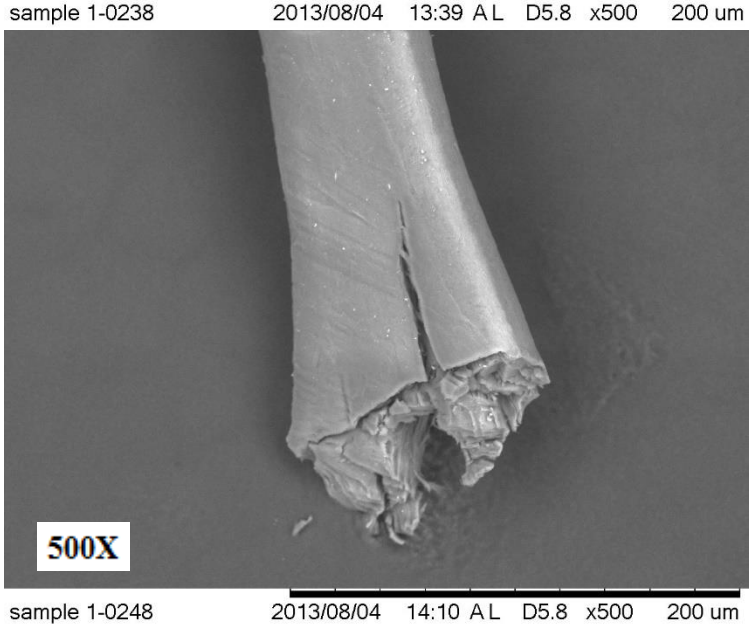




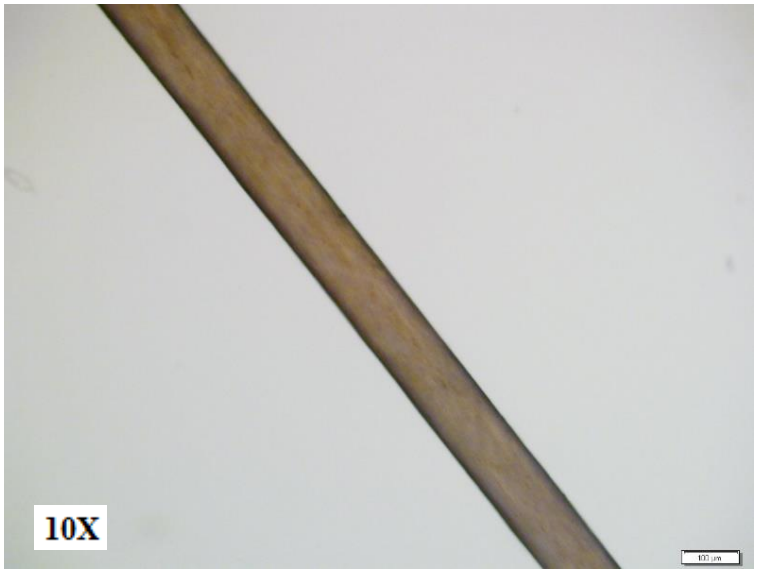



600X
sample 1-0231 2013/08/04 13:07 A L D5.8 x600 100 um


500X
sample 1-0238 2013/08/04 13:39 A L D5.8 x500 200 um


500X
sample 1-0248 2013/08/04 14:10 A L D5.8 x500 200 um

SAMPLE 6 – European Caucasian Weave
Transmitted Light Microscopy

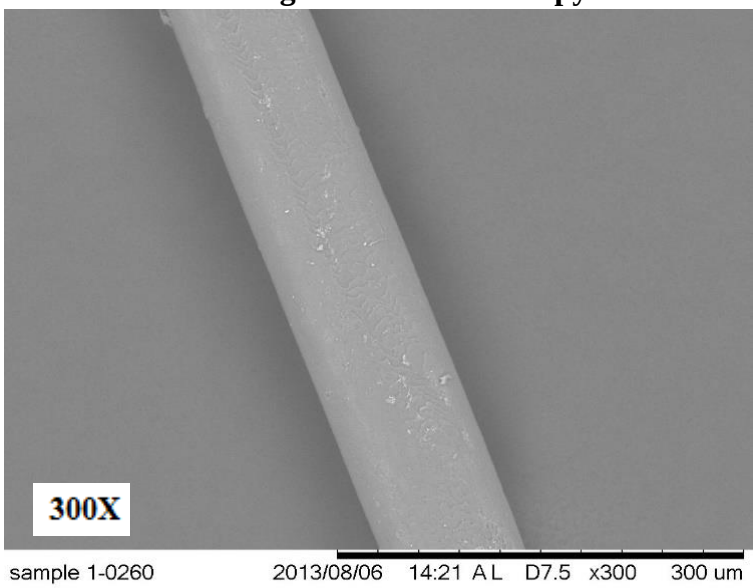


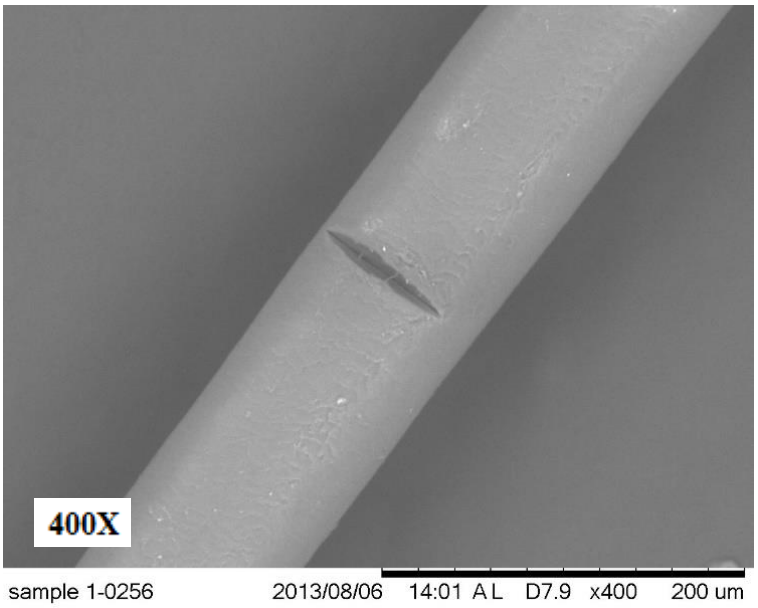

10X

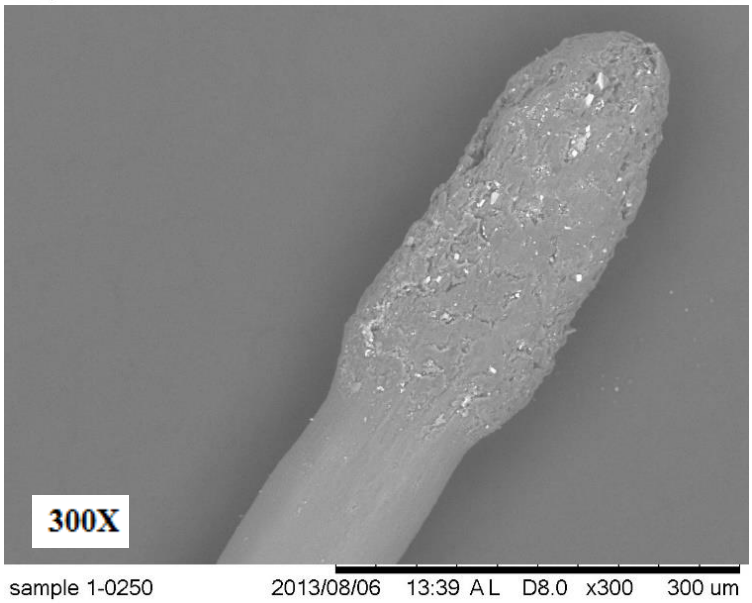

10X


20X

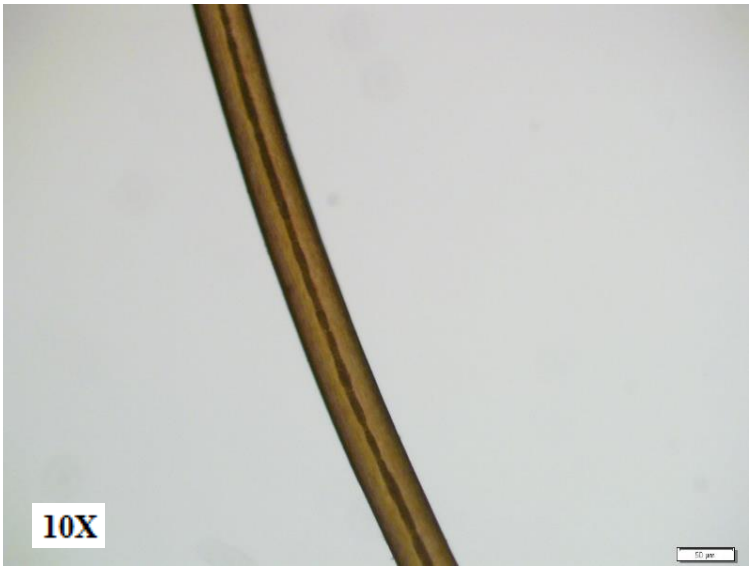
SAMPLE 6 – European Caucasian Weave
Scanning Electron Microscopy

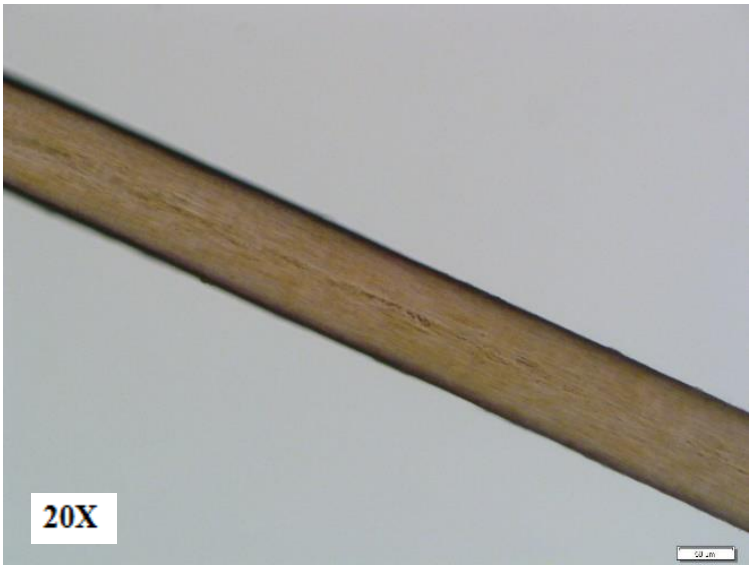


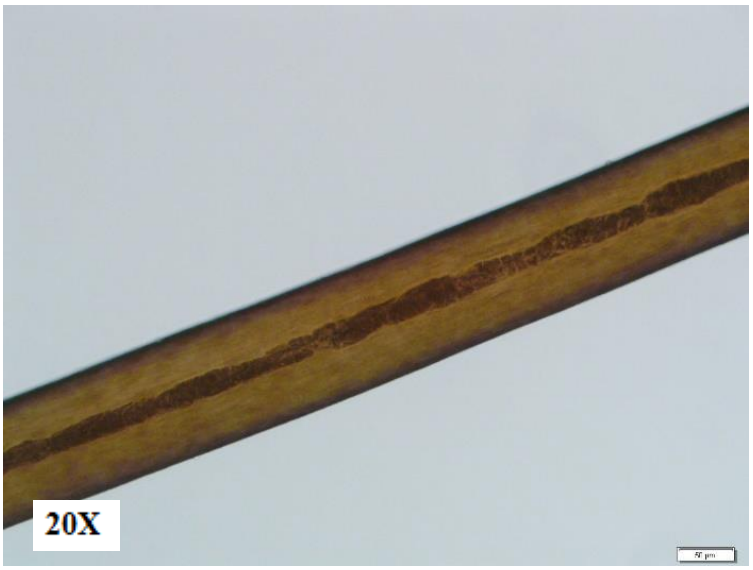


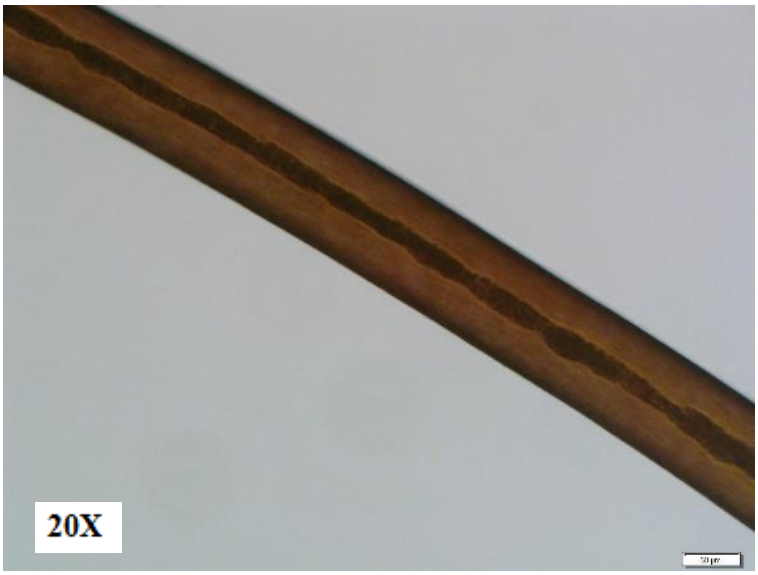


SAMPLE 7 – European Caucasian Weave
Transmitted Light Microscopy



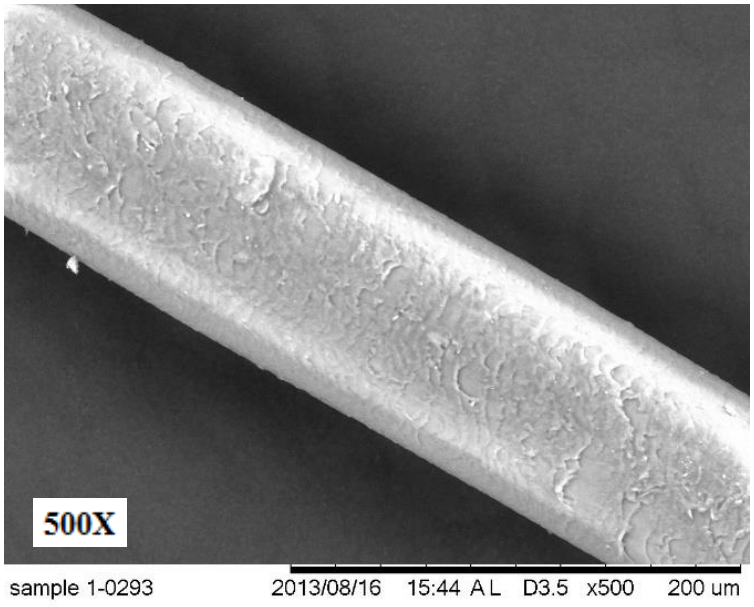


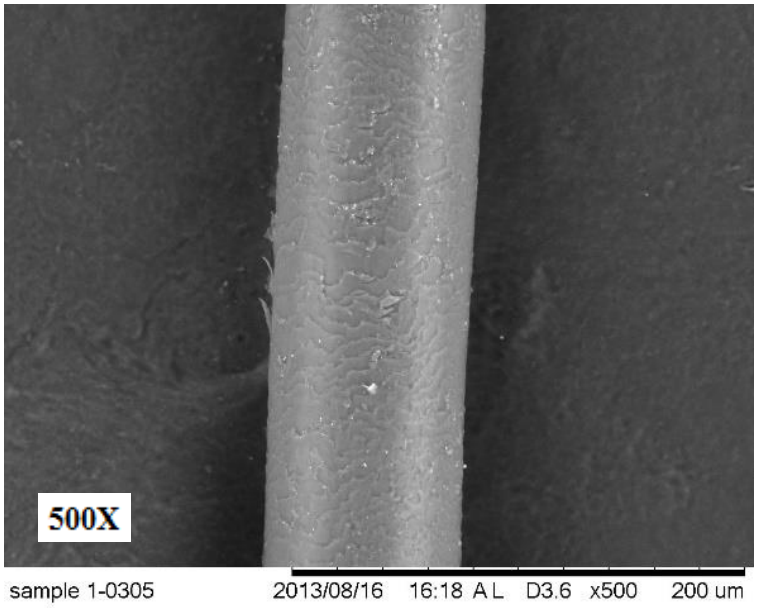

20X

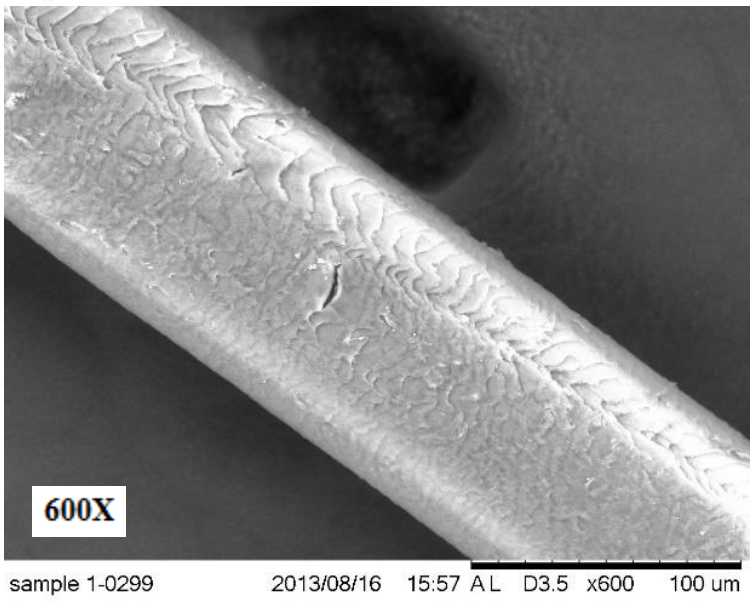

20X

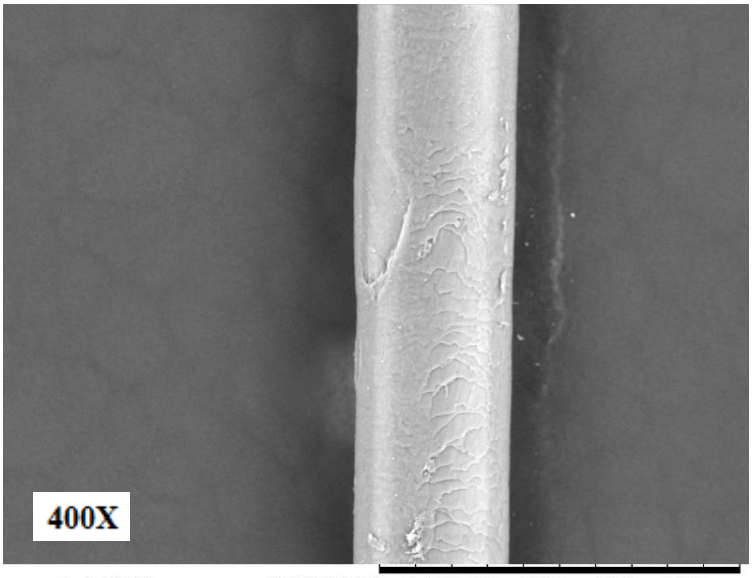

20X

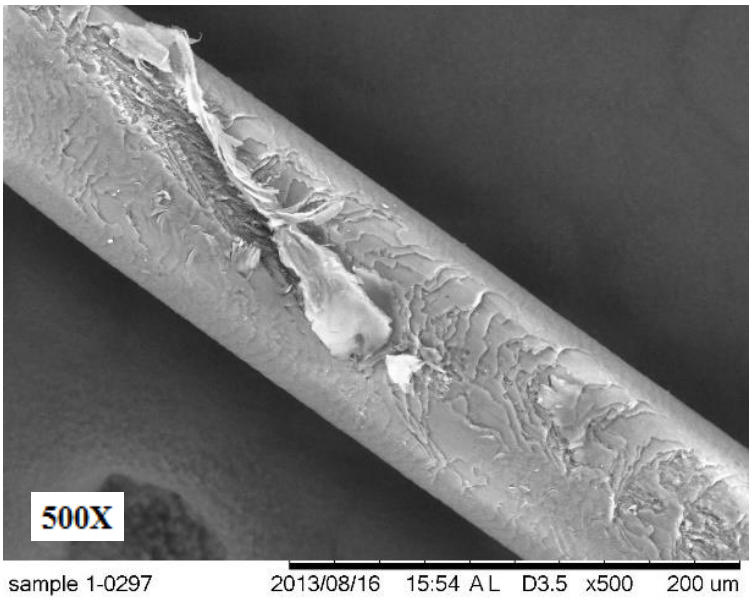
SAMPLE 7 – European Caucasian Weave
Scanning Electron Microscopy

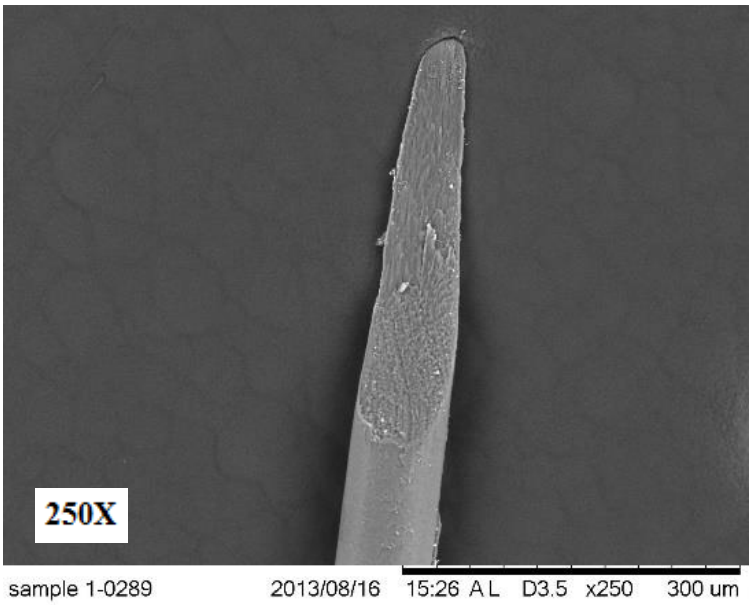




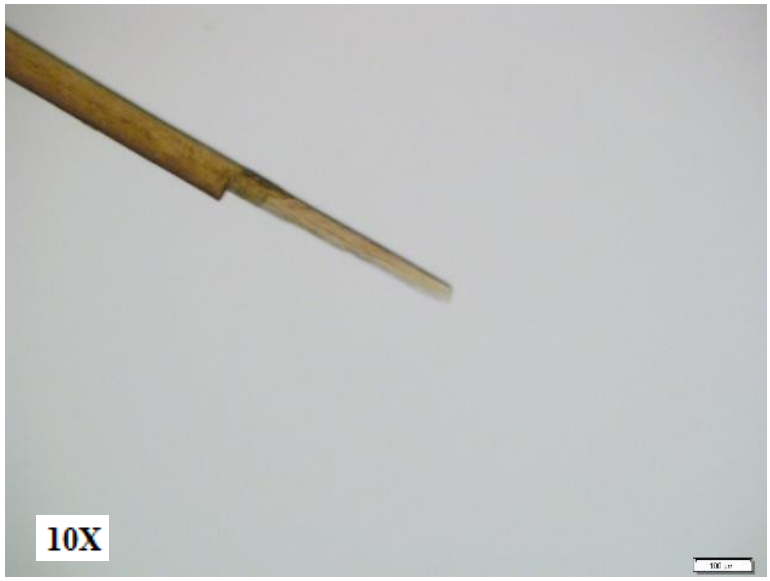


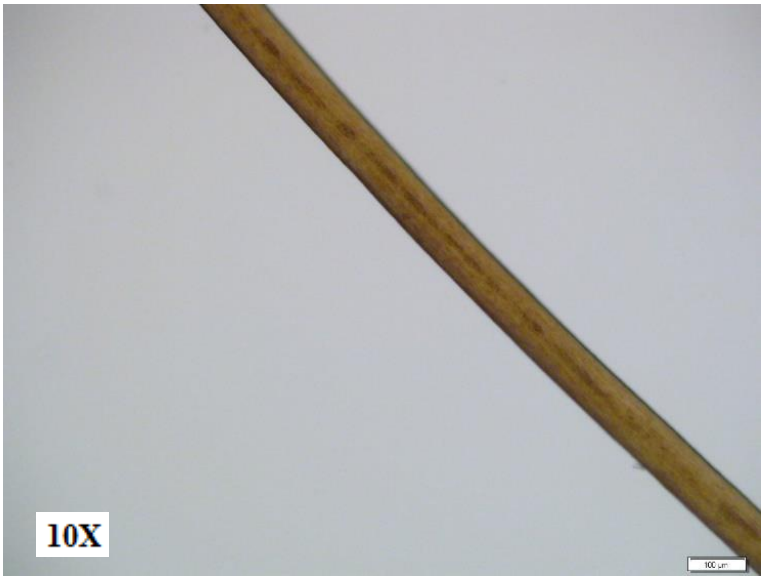

400X
sample 1-0281
2013/08/16 14:59 A L D3.5 x400 200 um

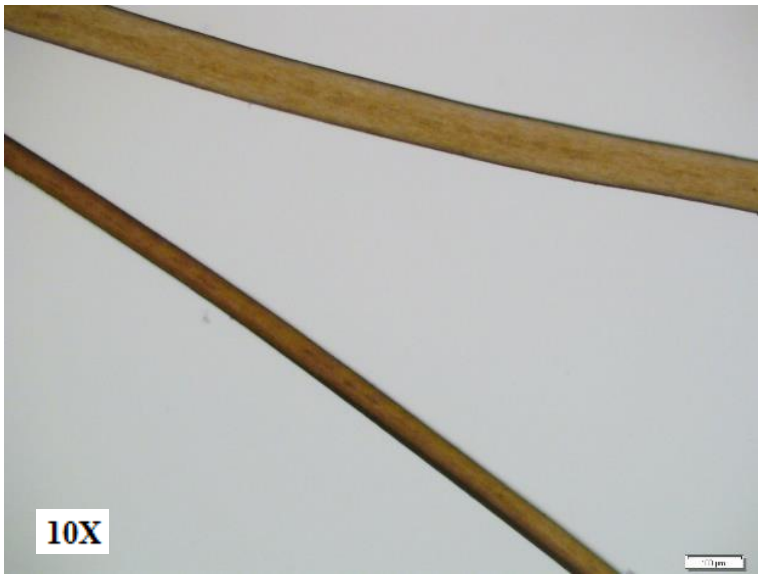

500X
sample 1-0297
2013/08/16 15:54 A L D3.5 x500 200 um


250X
sample 1-0289
2013/08/16 15:26 A L D3.5 x250 300 um

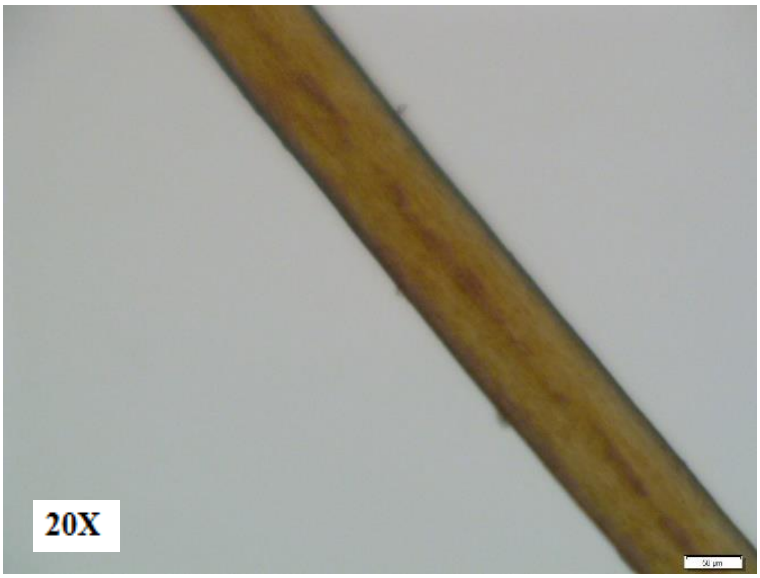
SAMPLE 8 – European Caucasian Weave
Transmitted Light Microscopy



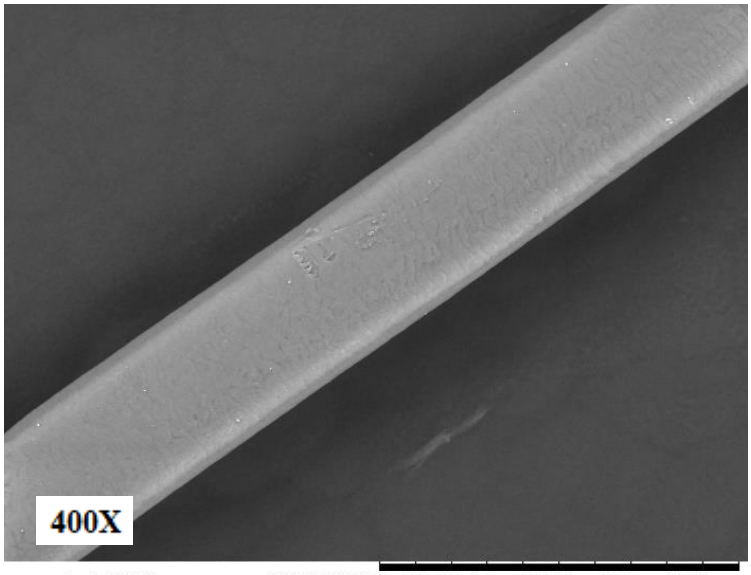



10X

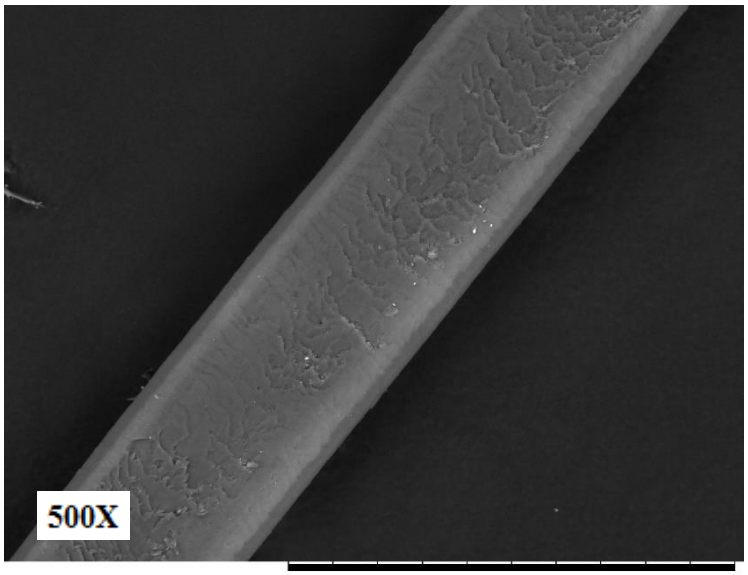

20X


20X

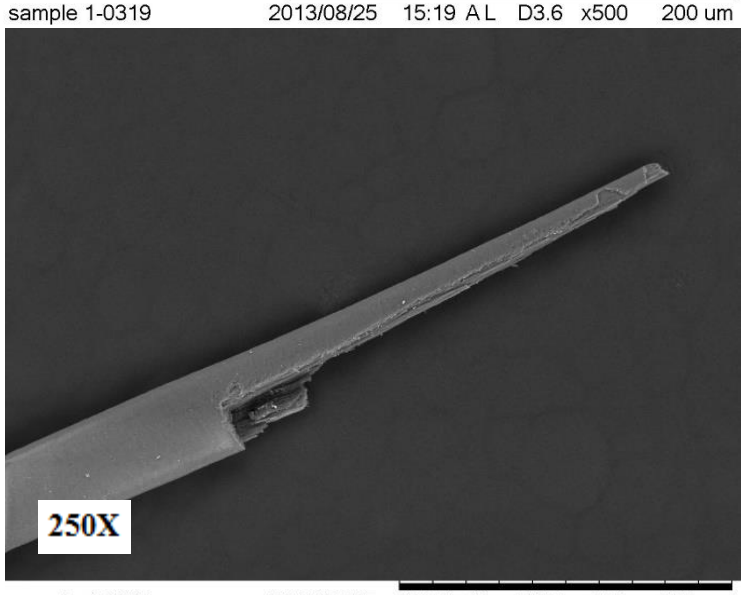
SAMPLE 8 – European Caucasian Weave
Scanning Electron Microscopy



sample 1-0340 2013/08/25 16:35 A L D3.5 x400 200 um

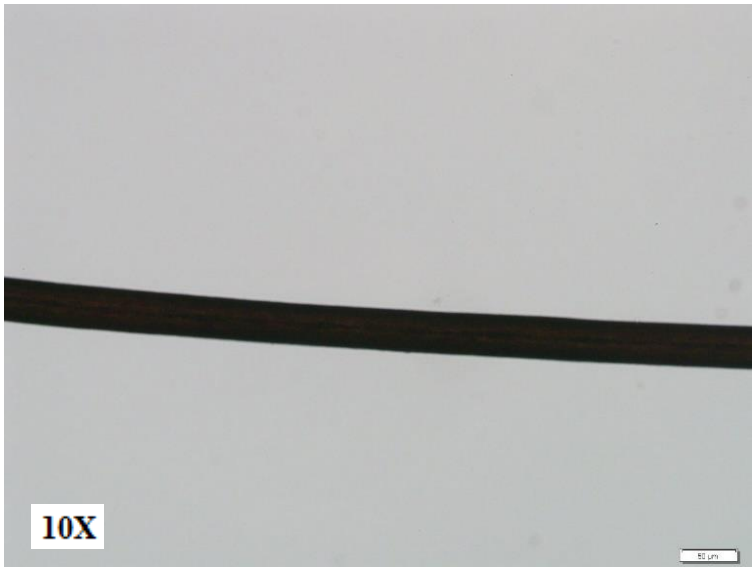


sample 1-0319 2013/08/25 15:19 A L D3.6 x500 200 um

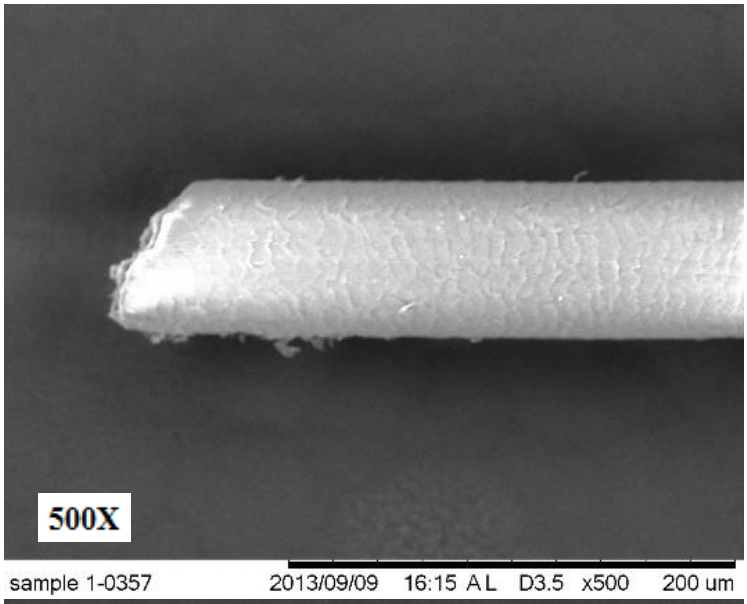


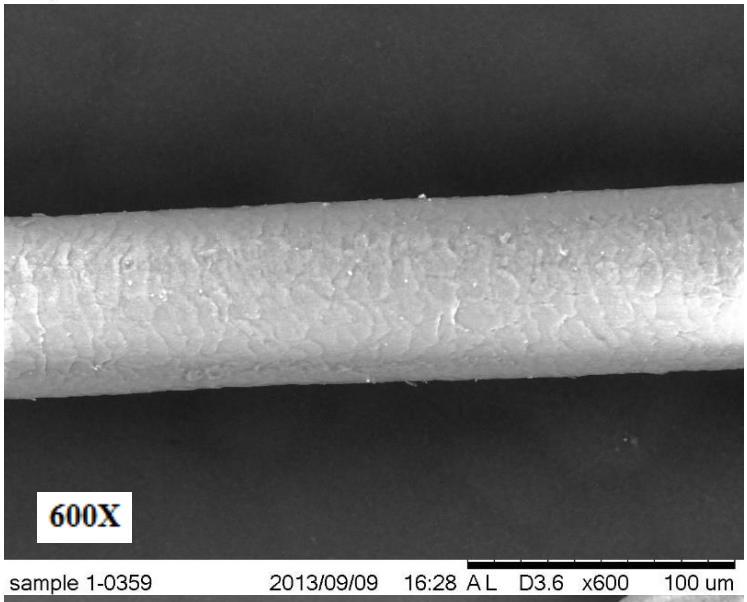
sample 1-0331 2013/08/25 15:50 A L D3.5 x250 300 um

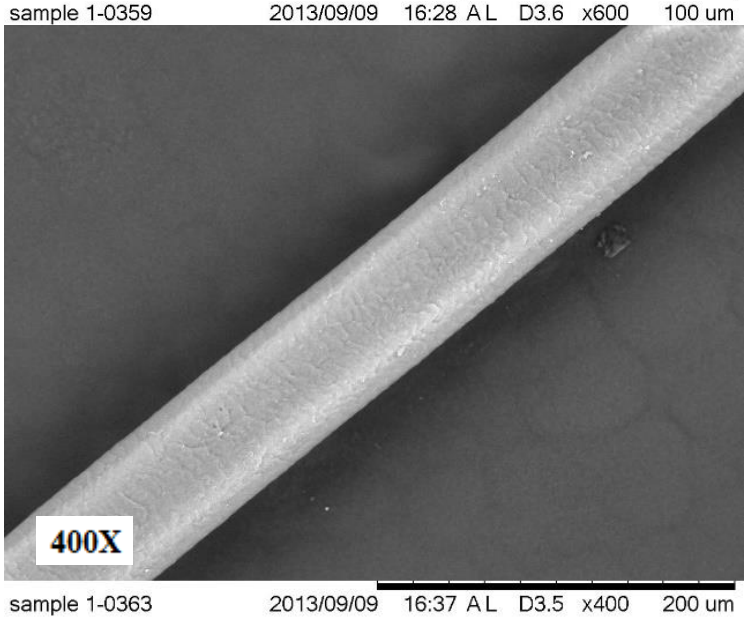
SAMPLE 11 – Que
Transmitted Light Microscopy

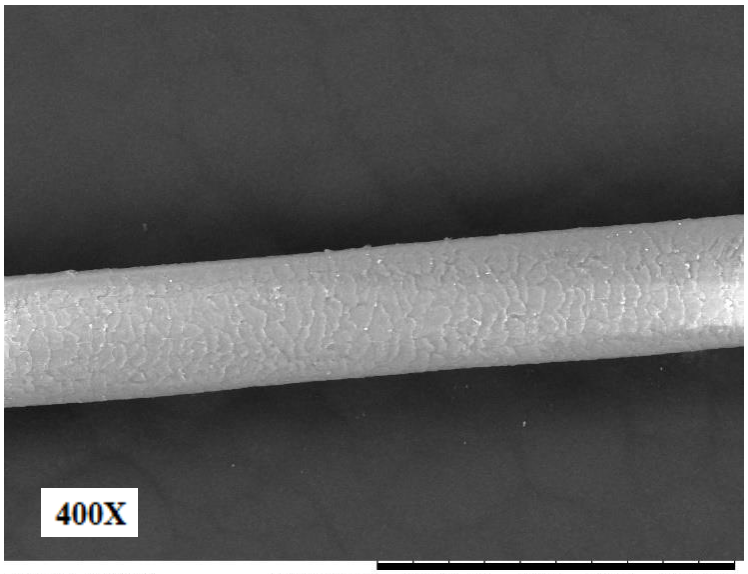


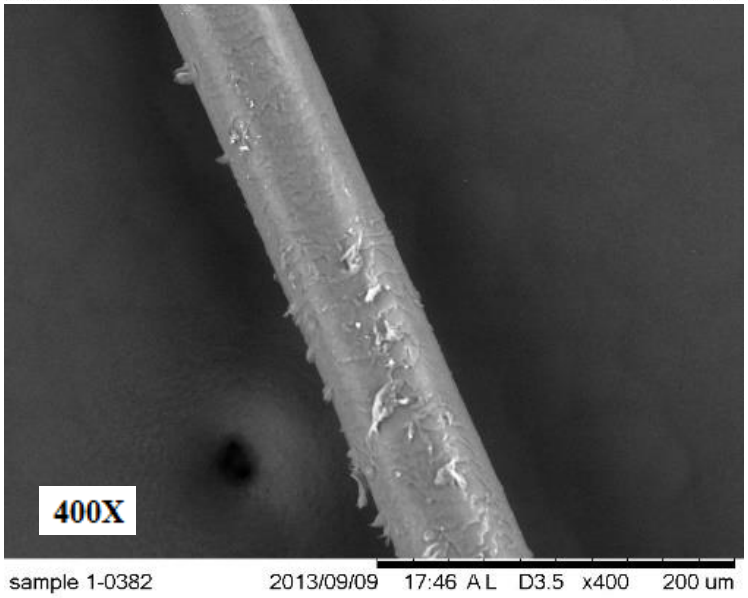
SAMPLE 11 – Que
Scanning Electron Microscopy

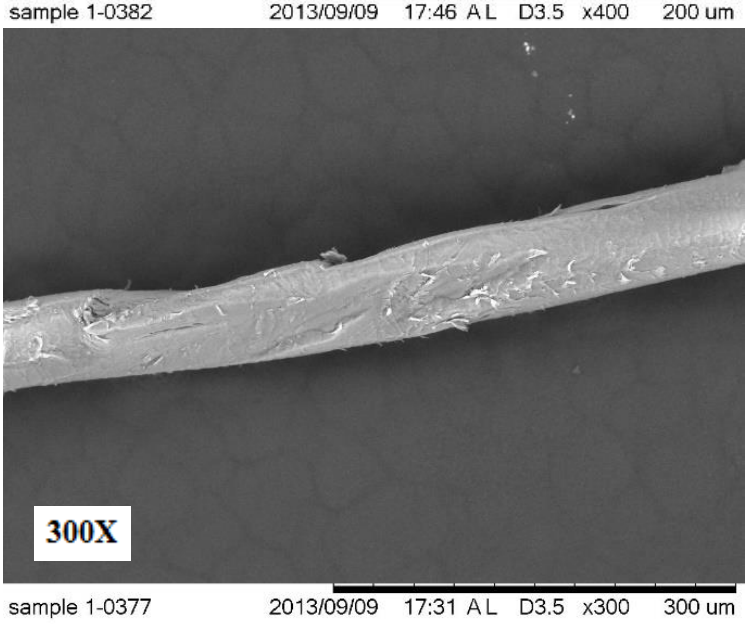




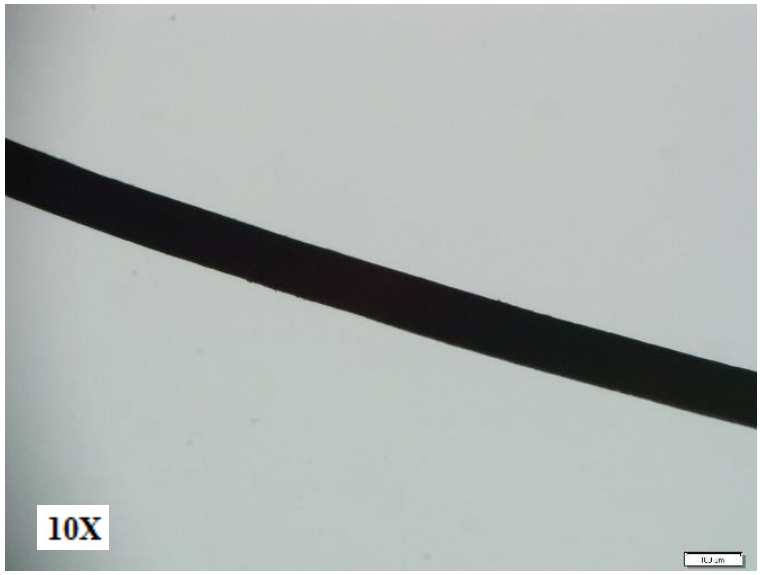


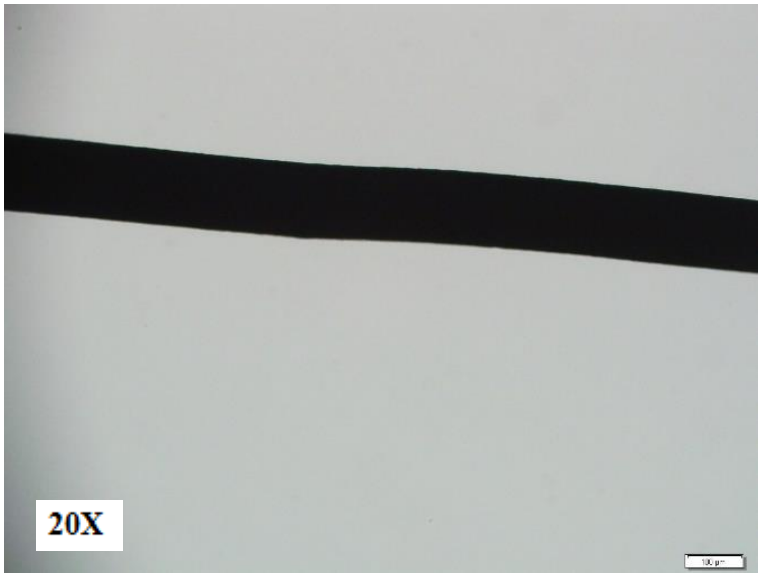

400X
sample 1-0360 2013/09/09 16:29 A L D3.6 x400 200 um

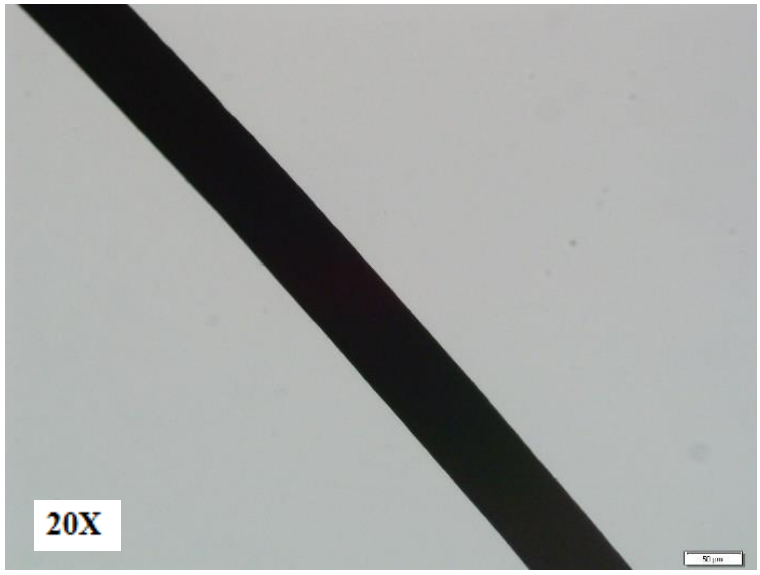

400X
sample 1-0382 2013/09/09 17:46 A L D3.5 x400 200 um


300X
sample 1-0377 2013/09/09 17:31 A L D3.5 x300 300 um

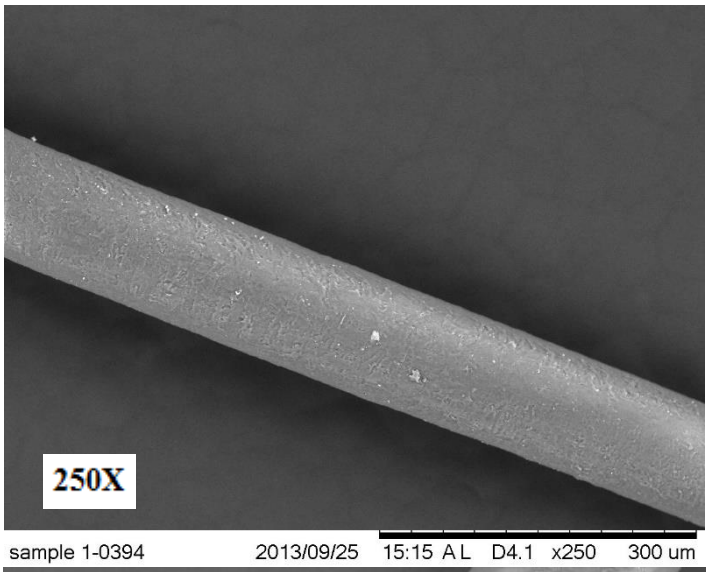
SAMPLE 12 – Que
Transmitted Light Microscopy

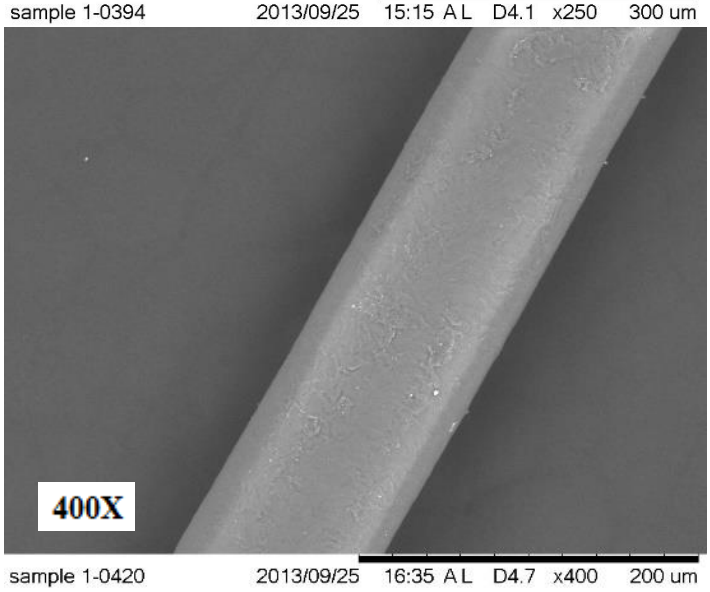


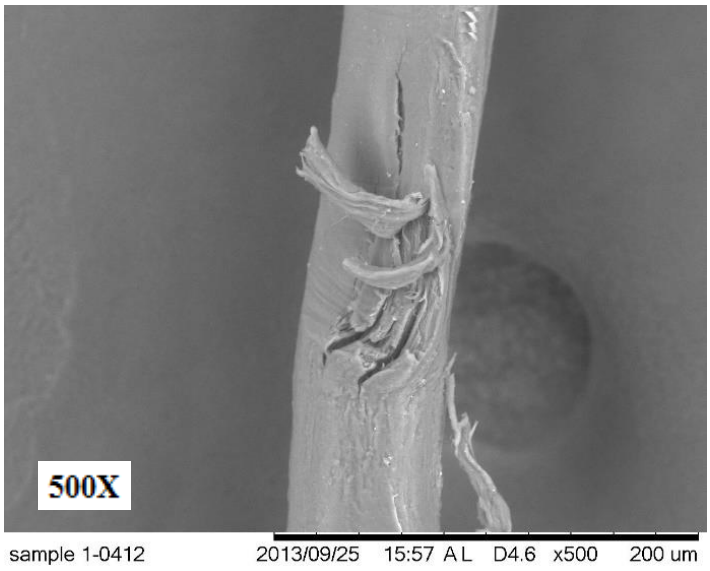




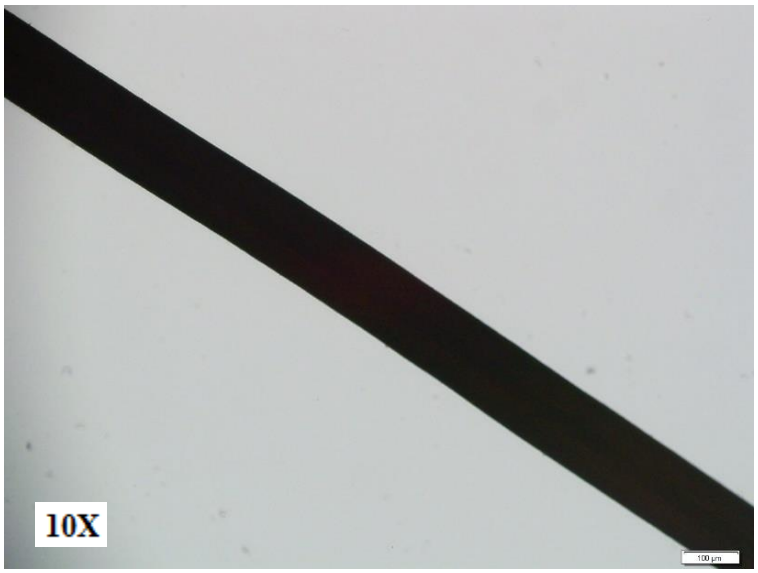
SAMPLE 12 – Que
Scanning Electron Microscopy

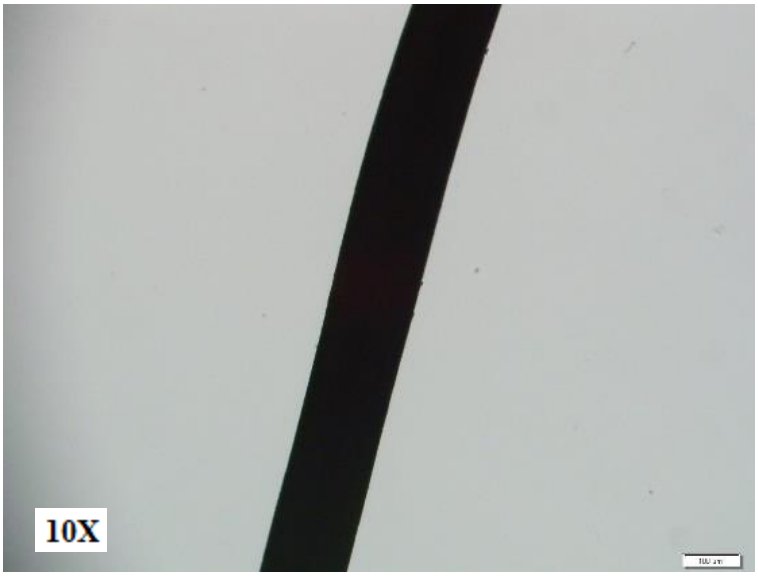


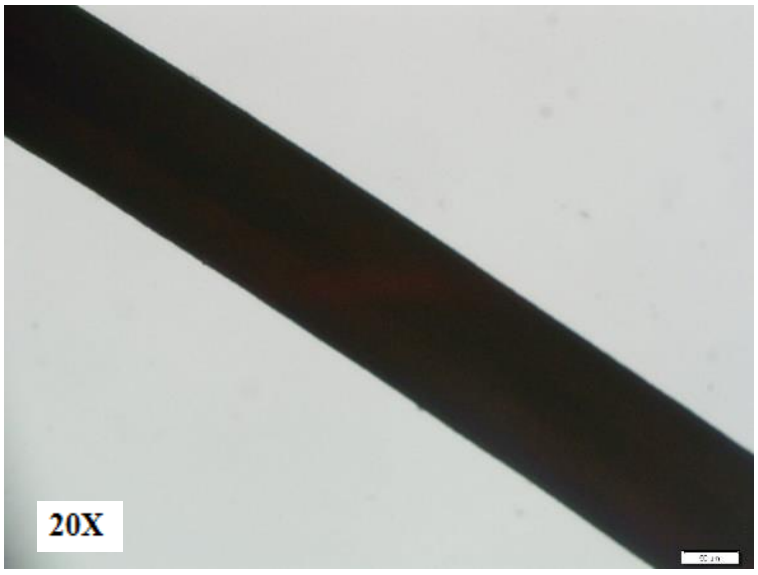




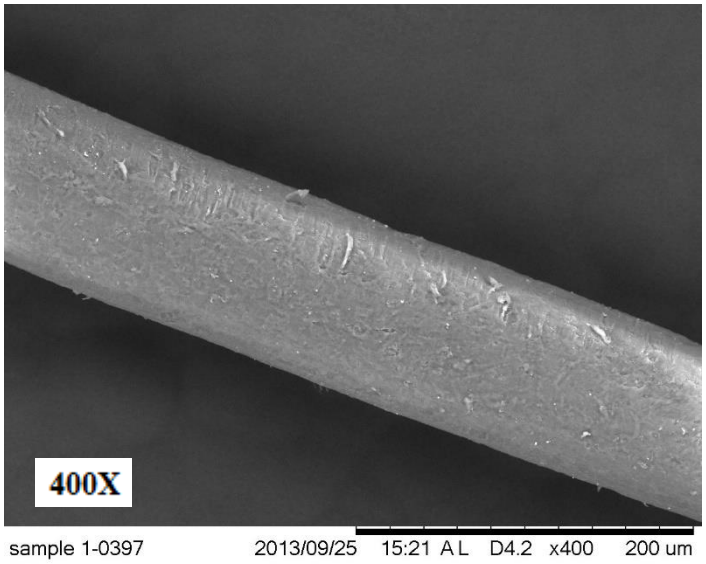
SAMPLE 13 – Que
Transmitted Light Microscopy

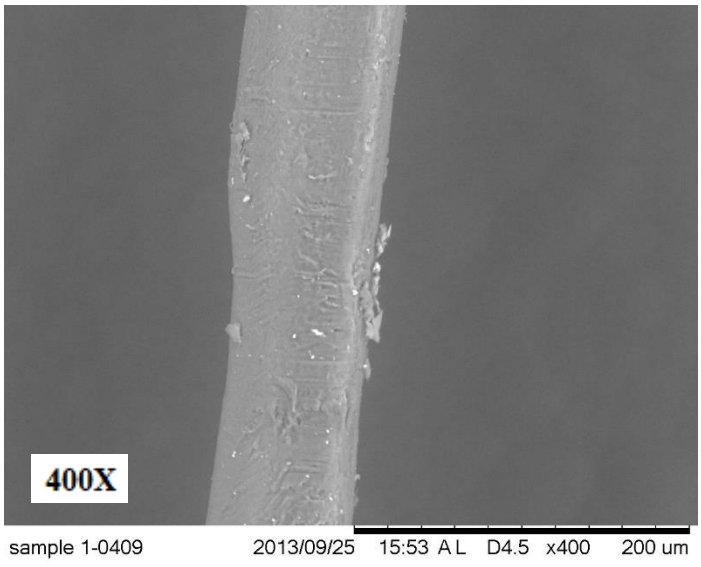


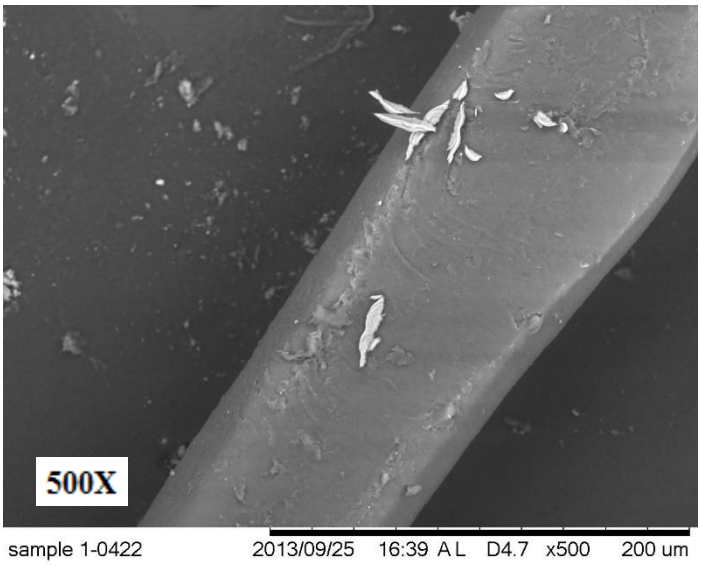




SAMPLE 13 – Que
Scanning Electron Microscopy

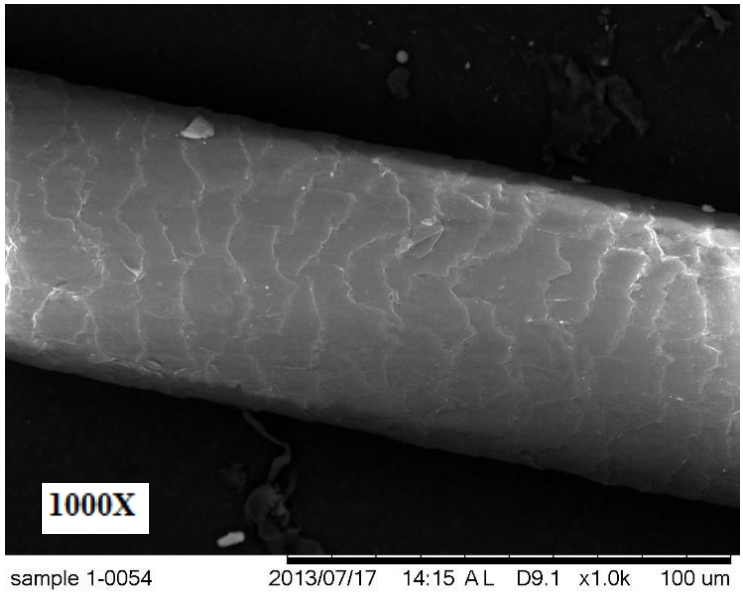


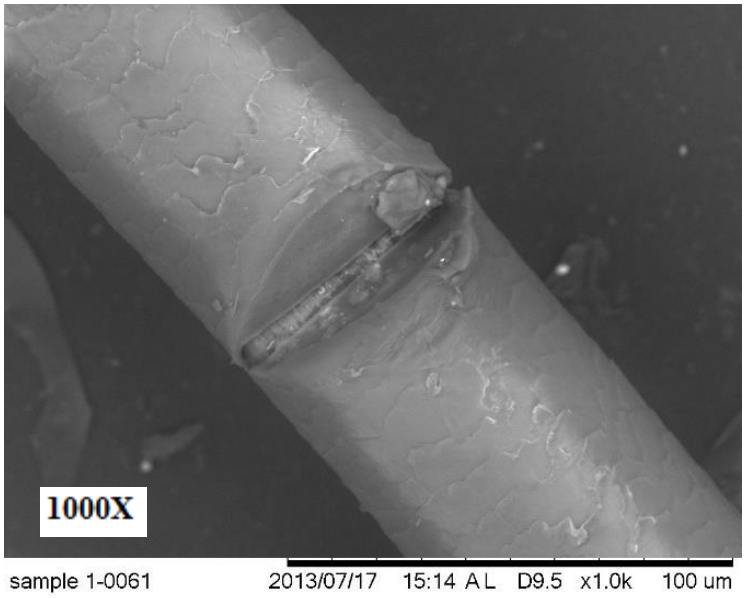


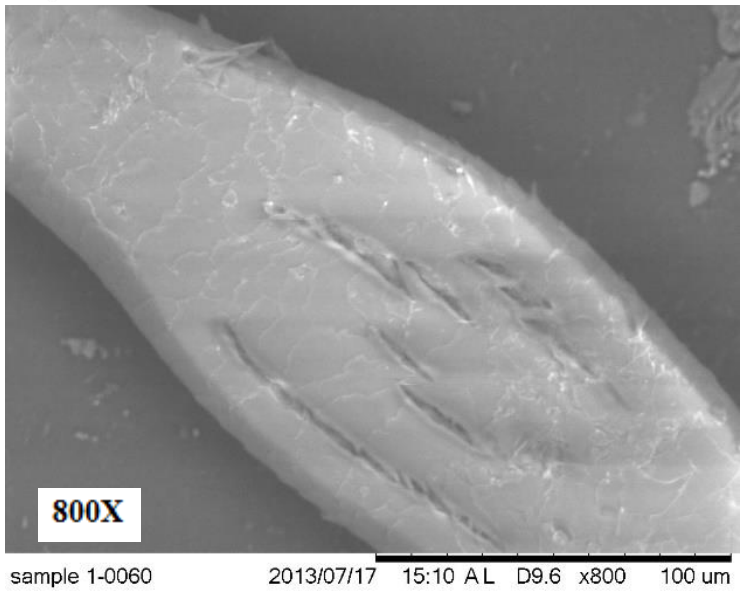


SAMPLE 16 – Caucasian Known
Transmitted Light Microscopy

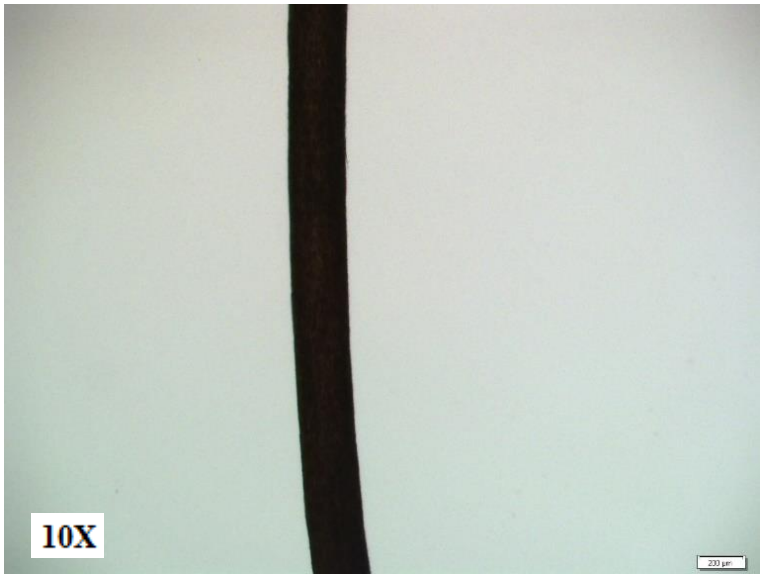
SAMPLE 16 – Caucasian Known
Scanning Electron Microscopy

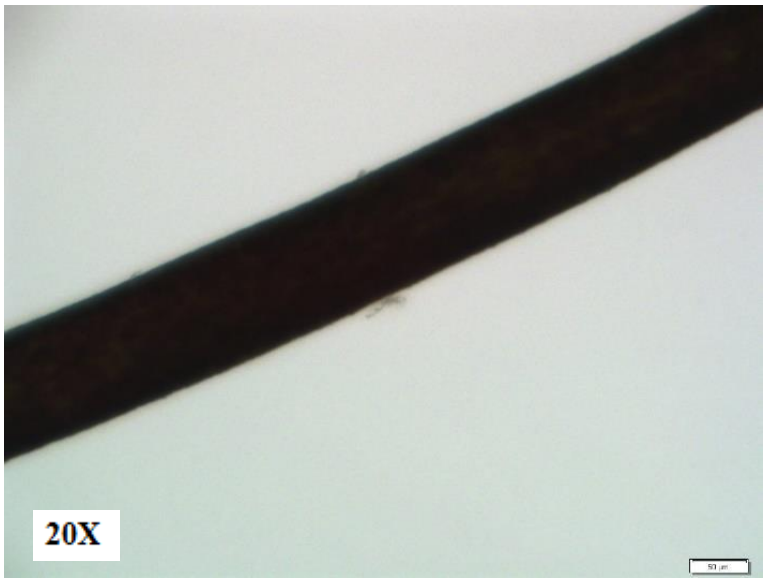


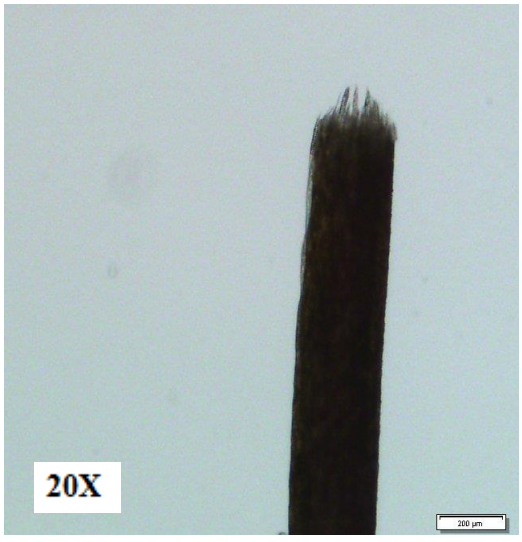




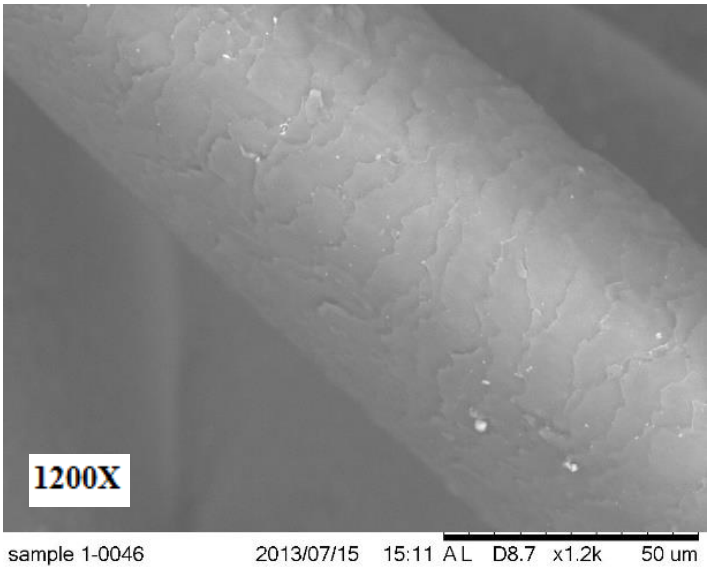
SAMPLE 17 – Negroid Known
Transmitted Light Microscopy

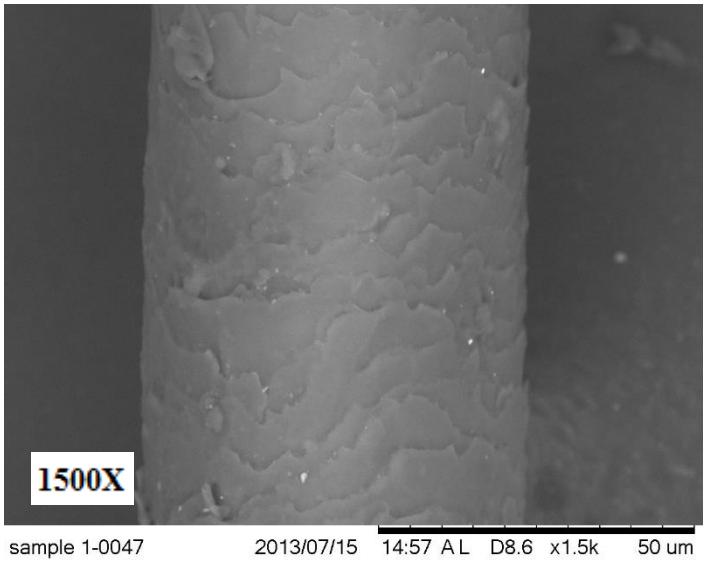




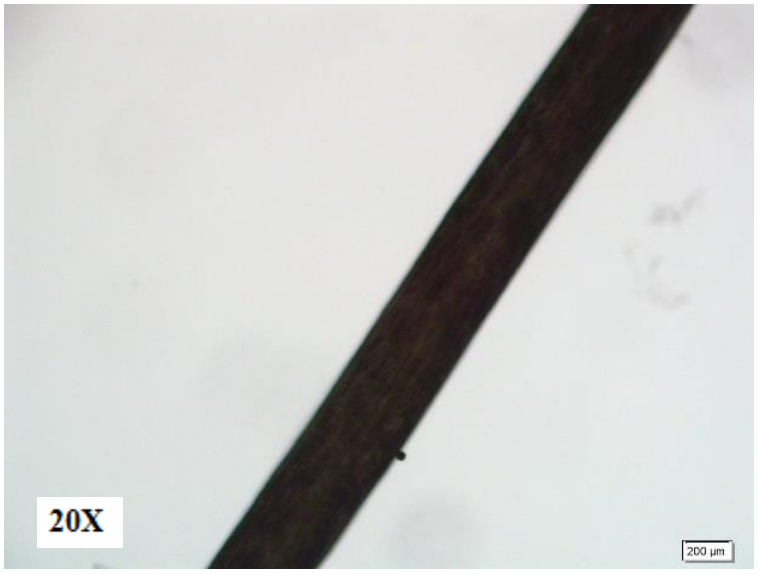


SAMPLE 17 – Negroid Known
Scanning Electron Microscopy





SAMPLE 18 – Mongoloid Known
Transmitted Light Microscopy

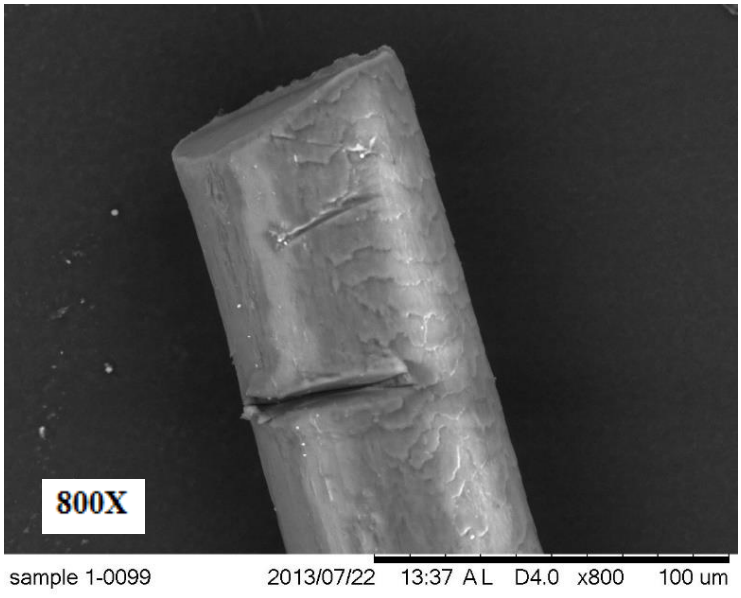


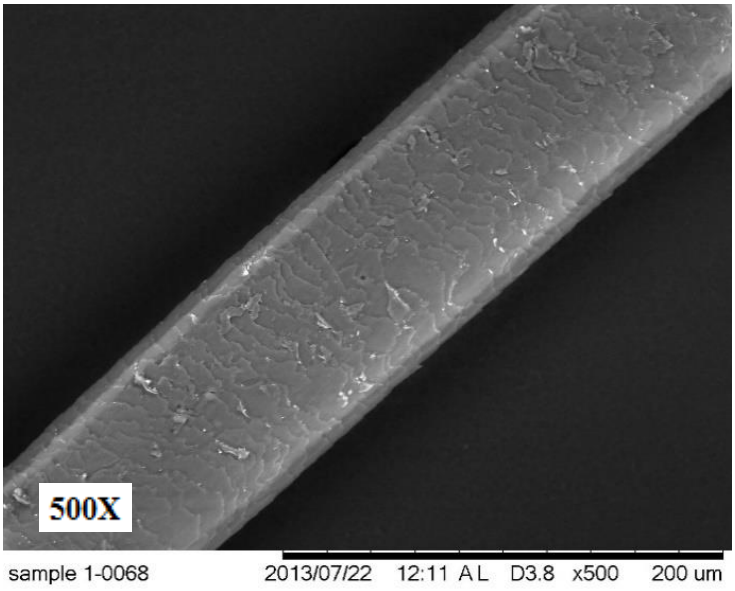

10X
100 µm

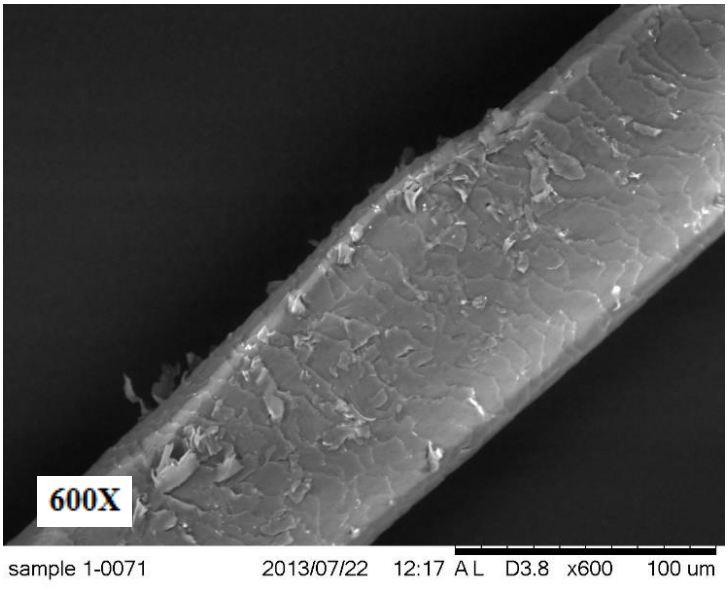

10X
100 µm

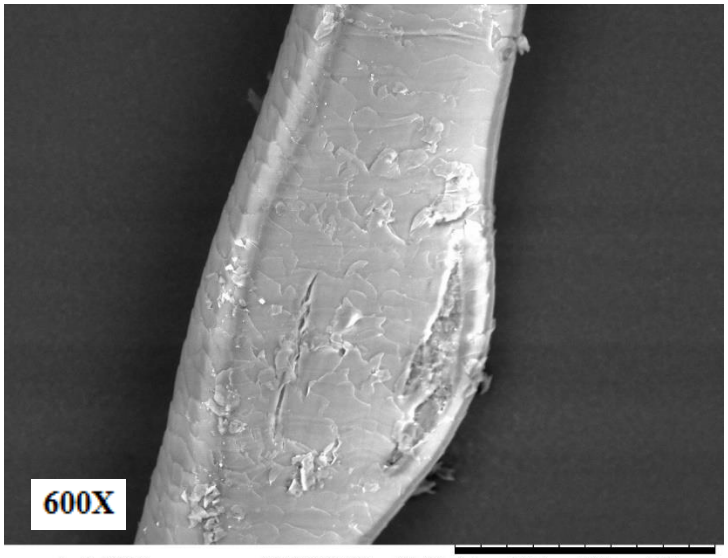

20X
200 µm

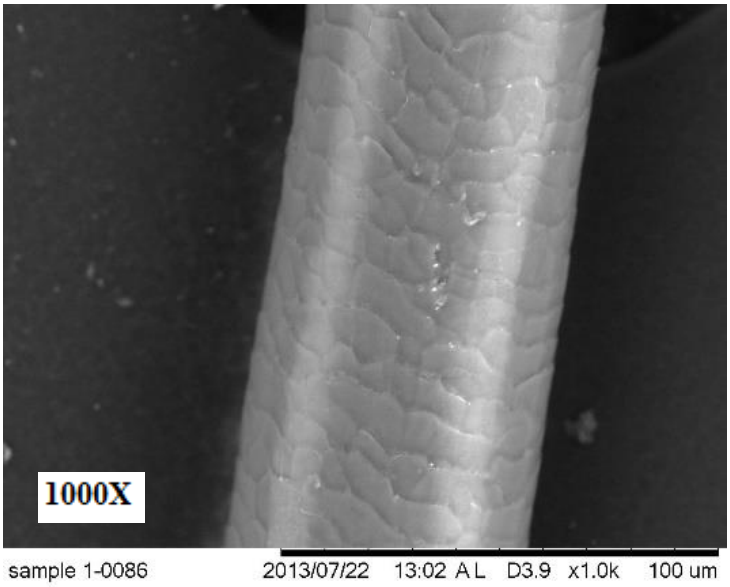
SAMPLE 18 – Mongoloid Known
Scanning Electron Microscopy

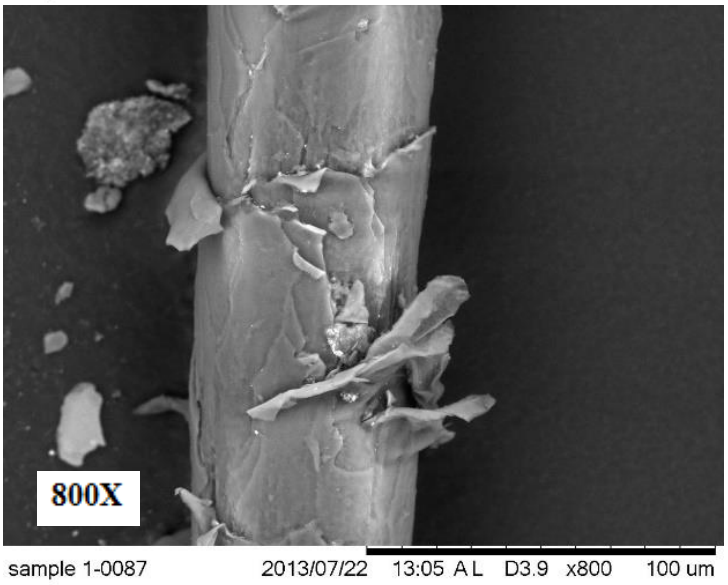


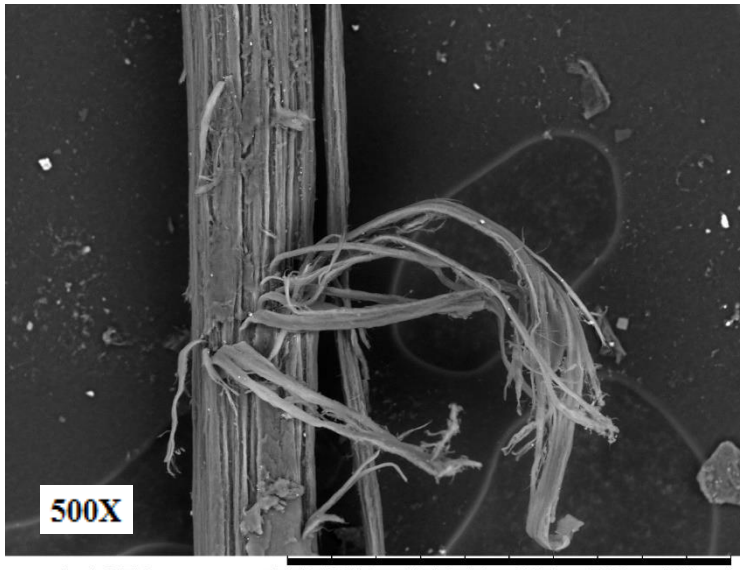


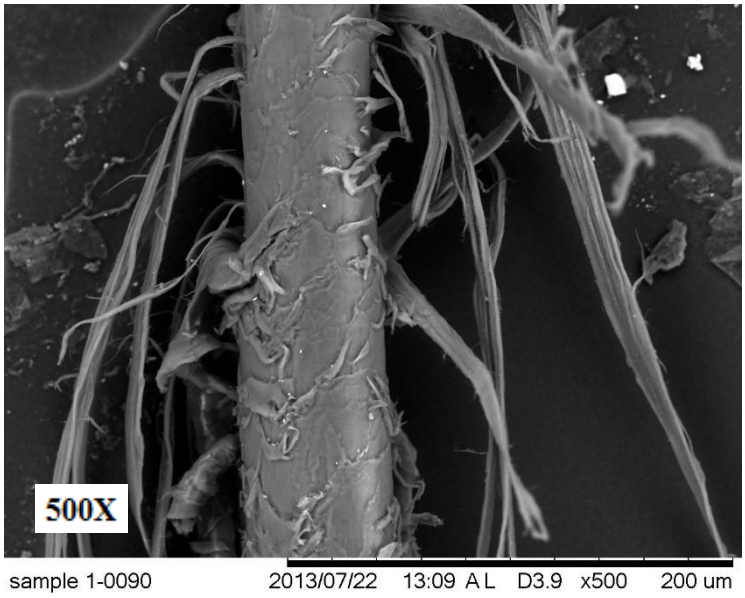


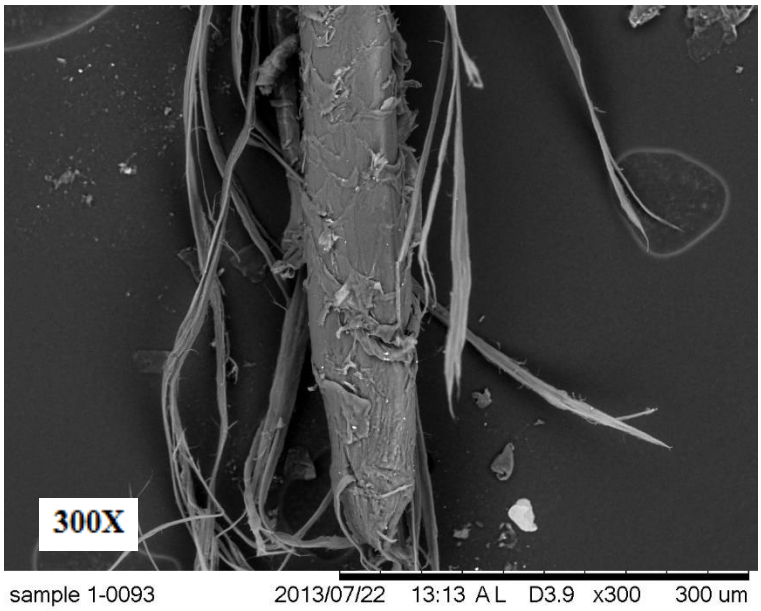

600X
sample 1-0085 2013/07/22 13:00 A L D3.9 x600 100 um


1000X
sample 1-0086 2013/07/22 13:02 A L D3.9 x1.0k 100 um

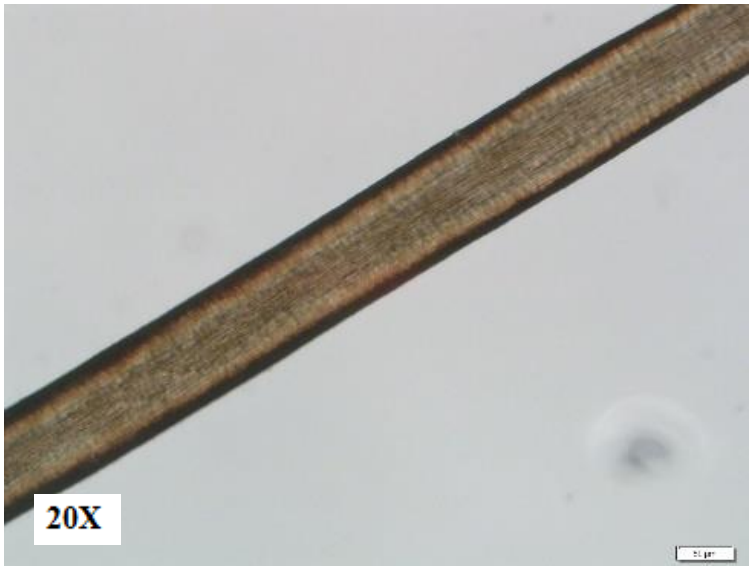

800X
sample 1-0087 2013/07/22 13:05 A L D3.9 x800 100 um

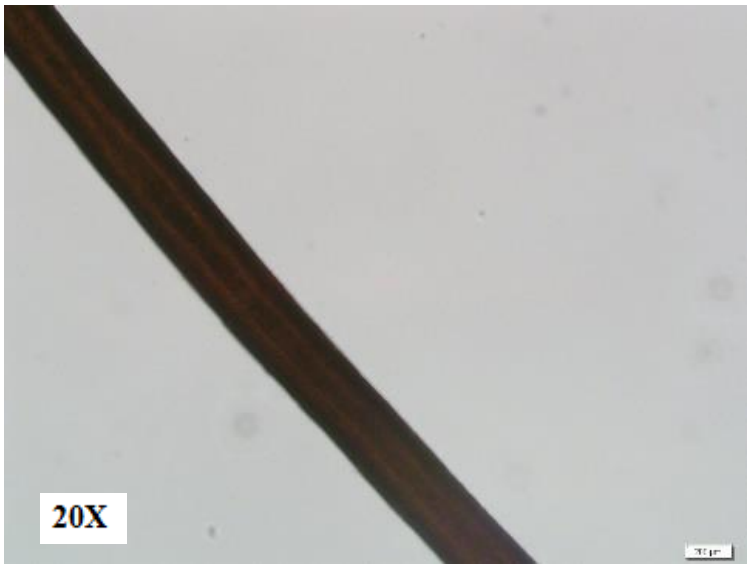

500X
sample 1-0088 2013/07/22 13:06 A L D3.9 x500 200 um

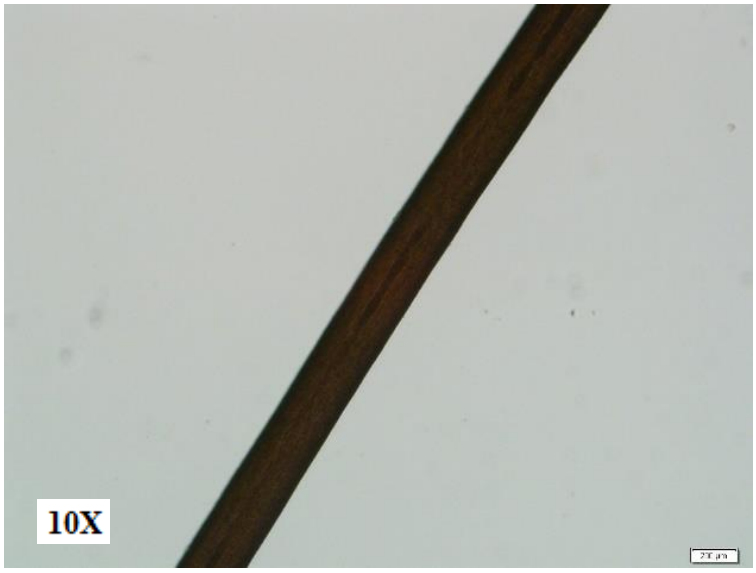

500X
sample 1-0090 2013/07/22 13:09 A L D3.9 x500 200 um

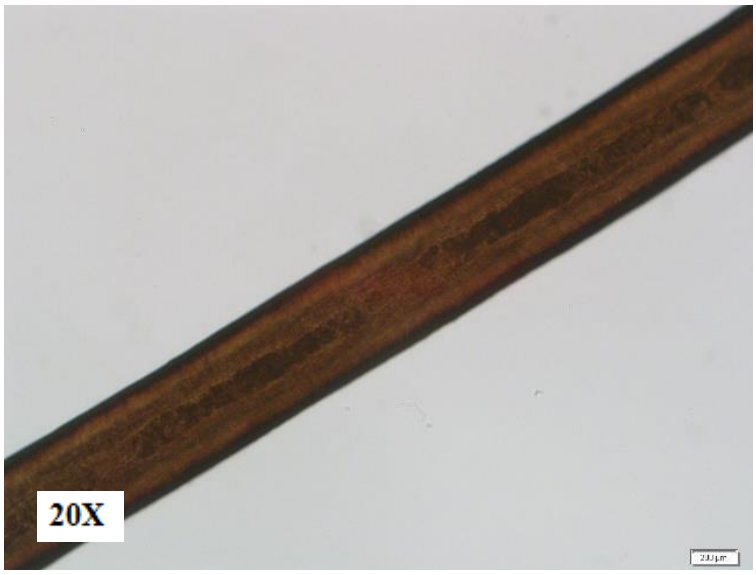

300X
sample 1-0093 2013/07/22 13:13 A L D3.9 x300 300 um

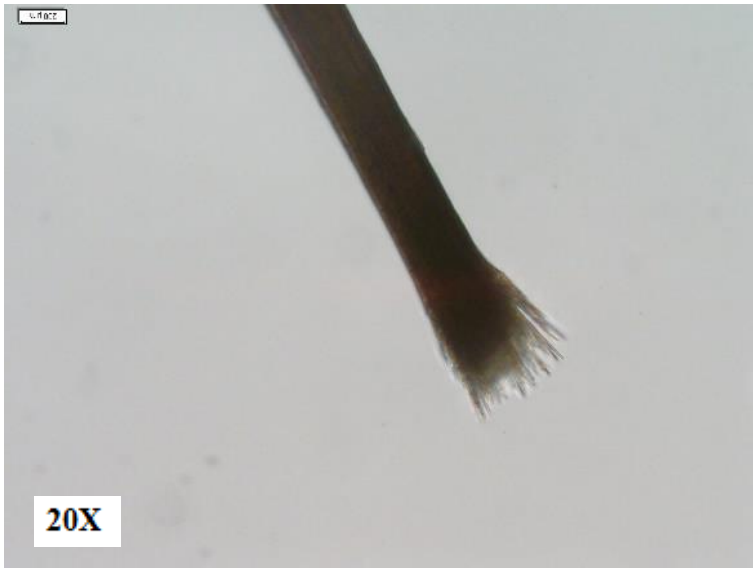
SAMPLE 19 – Caucasian Dyed Known
Transmitted Light Microscopy



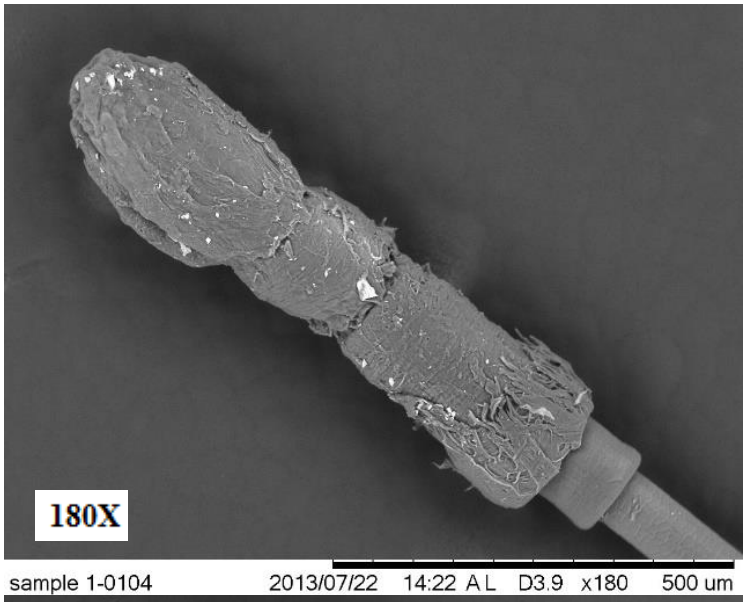


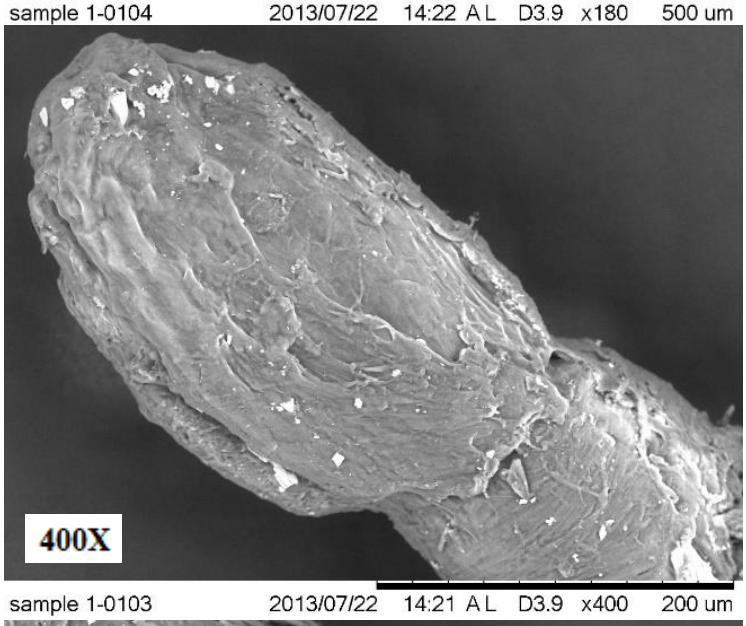

10X

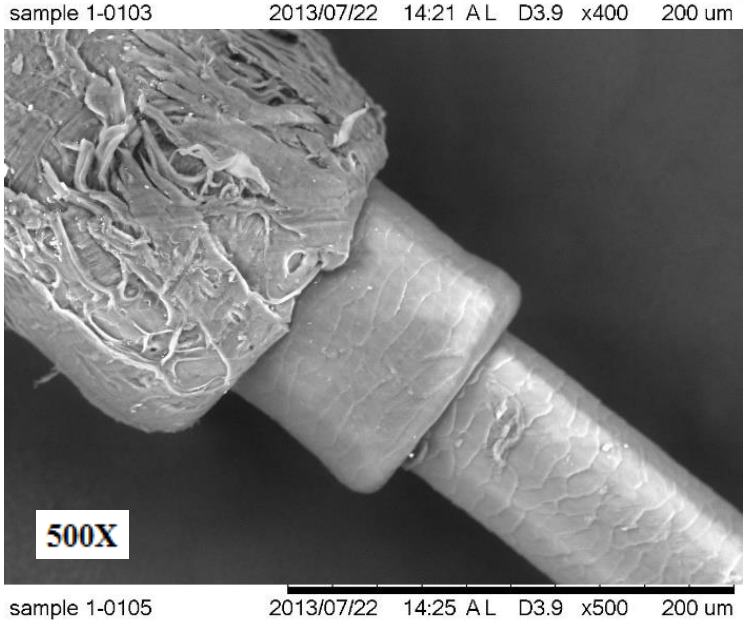

20X


20X

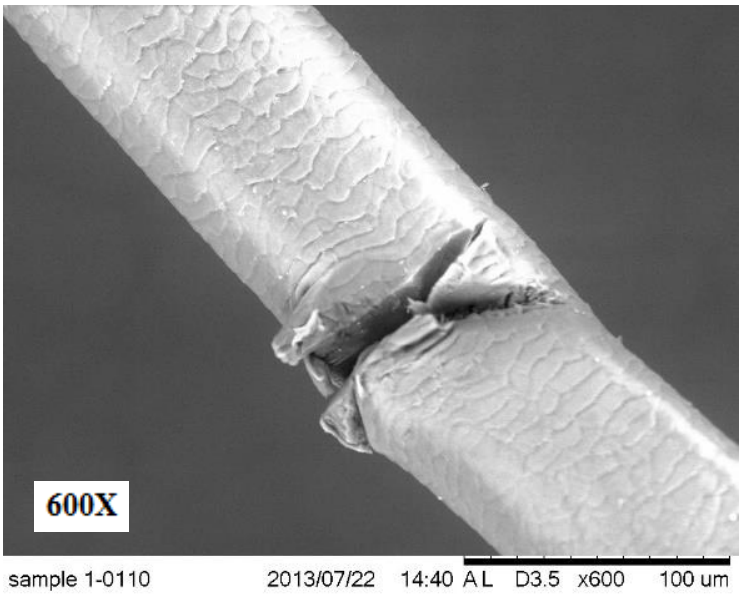
SAMPLE 19 – Caucasian Dyed Known
Scanning Electron Microscopy

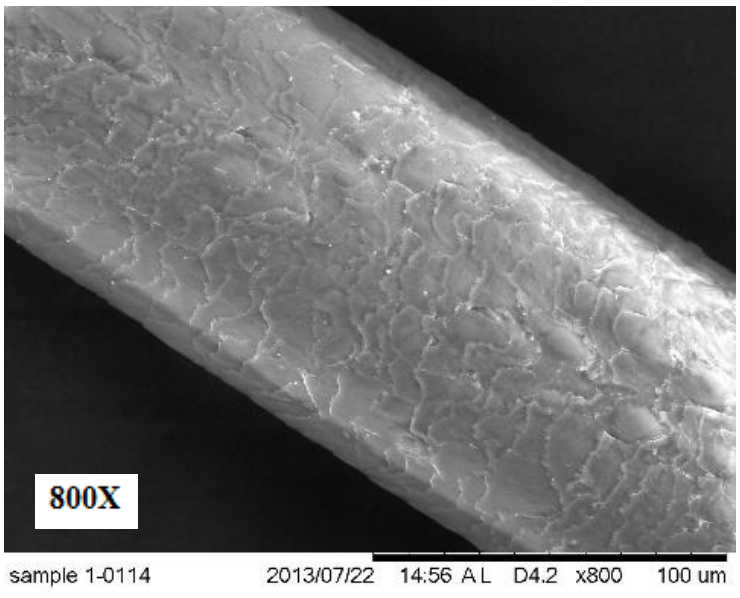


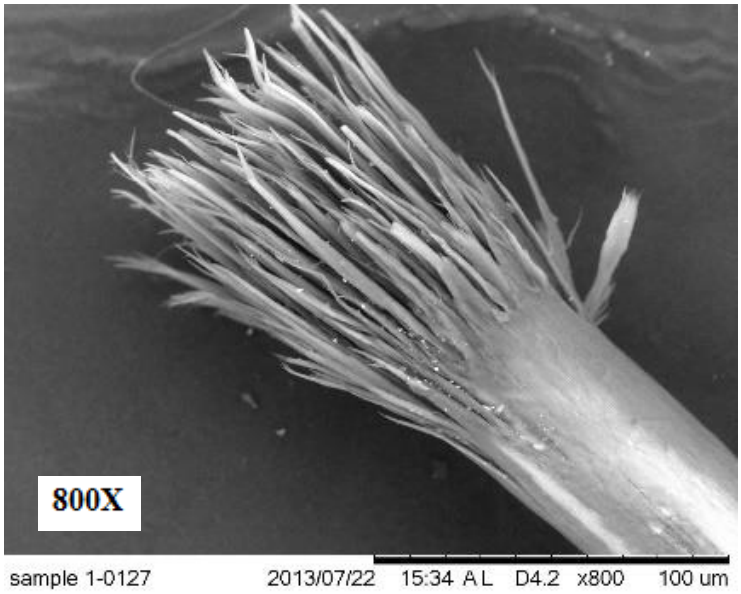





400X
sample 1-0108 2013/07/22 14:36 A L D3.7 x400 200 um


600X
sample 1-0110 2013/07/22 14:40 A L D3.5 x600 100 um


800X
sample 1-0114 2013/07/22 14:56 A L D4.2 x800 100 um


800X
sample 1-0127
2013/07/22 15:34 AL D4.2 x800 100 um

Appendix B

mtDNA Sequences of Processed Human Hair Extensions and Mongoloid Known Sample 18 (positive control)

Appendix B: mtDNA Sequences of Processed Human Hair Extensions and Mongoloid Known Sample 18 (positive control)

Sample 1 - Remy XQ
Number of Base Pairs: 334
Region Sequenced: HV2 of mtDNA Genome

CTGGGGGGTGTGCACGCGATAGCATTGCGAGACGCTGGGAGCCGTGAGCACCCTAT
GTCGCAGTATCTGTCTTTGATTCCTGCCTCATTCCATTATTTATCGCACCTACGTTCA
ATATTACAGGCGAACATACTTATAAAGTGTGTTAATTAATTAATGCTTGTAGGACAT
AATAATAACAATTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAA
AATTTCCACCAAACCCCCCCCTCCCCCCGCTTCTGGCCACAGCACTTAAACACATCT
CTGCCAAACCCCAAAAACAAAGAACCCTAACACCAGCCTAACCAGAGGA

Sample 2 - Remy XQ
Number of Base Pairs: 285
Region Sequenced: HV2 of mtDNA Genome

GTGCCGCGATAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCT
GTCTTTGATTCCTGCCTCATTCCATTATTTATCGCACCTACGTTCAATATTACAGGCG
AACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAACAA
TTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTCCACCAA
ACCCCCCCCTCCCCCCGCTTCTGGGCACAGCACTTAAACACATCTCTGCCAAACCCC

Sample 3 - Remy XQ
Number of Bases: 327
Region Sequenced: HV2 of mtDNA Genome

TGTGCACGCGATAGCATTGCGAGACGCTGGAGCCGGTAGCACCCTATGTCGCAGTAT
CTGTCTTTGATTCCTGCCTCATTCCATTATTTATCGCACCTACGTTCAATATTACAGG
CGAACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAAC
AATTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTCCACC
AAACCCCCCCCTCCCCCCGCTTCTGGCCACAGGGACTTAAACACATCTCTGCCAAAC
CCCAAAAACAAAGAACCCTAACACCAGGCCTAACCAGAAGG

Sample 7 – European Caucasian Weave (ECW)
Number of Bases: 290
Region Sequenced: HV2 of mtDNA genome

GCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCATTCCATTATTTA
TCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATT
AATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCGCTTTCCACACA
GACATCATAACAAAAAATTTCCACCAAACCCCCCCCTCCCCCCGCTTCTGGGCACAG
CACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACCAGCCTAA
CCAGA

Sample 8 – European Caucasian Weave (ECW)
Number of Bases: 309
Region Sequenced: HV2 of mtDNA genome

TGTGCCGCGATAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCT
GTCTTTGATTCCTGCCTCATCCTATTATTTATCGCACCTACGTTCAATATTACAGACG
AACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAACAA
TTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTTCACCAA
ACCCCCCCCCTCCCCCCGCTTCTGGTCACAGGCACTTAAACACATCTCTGCCAAACCC
CAAAAACAAAGAACCCTAACACC

Sample 12 – Que
Number of Bases: 303
Region Sequenced: HV2 of mtDNA genome

ACGCTGGAAGCCGGTTAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCAT
TCCATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAGTGT
GTTAATTAATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCG
CTTTCCACACAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCTCCCCCCGCT
TCTGGCCACAGGCACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTA
ACACCAGCCTAACCAGAA

Sample 13 - Que
Number of Bases: 243
Region Sequenced: HV2 of mtDNA genome

GTGTTTAGTGCTGTGCACGCGATAGCATTGCGAGGACGCTGGAGCCGGAGCACCCT
ATGTCGCAGTATCTGTCTTTGATTCCTGCCTCATTCCATTATTTATCGCACCTACGTTC
AATATTACAGGCGAACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGAC
ATAATAATAACAATTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAA
AAATTTCCACCAAA

Sample 18 – Mongoloid Known (positive control)
Number of Bases: 218
Region Sequenced: HV2 of mtDNA genome

GTCTTTGATTCCTGCCTCATTCCATTATTTATCGCACCTACGTTCAATATTACAGGCG
AACATACTTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAATAATAACAA
TTGAATGTCTGCACAGCCGCTTTCCACACAGACATCATAACAAAAAATTTCCACCAA
ACCCCCCCCCTTCCCCCCGCTTCTGGGCACAGGACTTAAACACATCT