Human DNA and RNA Quantification Historical Account #1

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This account focuses upon one of the most important steps in forensic biology: the ***quantification of the human DNA and RNA*** (nucleic acids) from biological samples. Determination of the quantity and quality of human nucleic acids extracted from biological evidence is important for several different reasons. First, different analytical methods require different nucleic acid amounts and quality for optimal results. Therefore, determining the quantity and quality facilitates both the selection of the analytical method as well as the amount of the extract to utilize.
Second, the utilization of the most judicious amount of the extract permits the preservation of as much of the evidence as possible for retesting or future testing with newer more powerful molecular methods. Third, the quantitative and qualitative evaluations facilitates a more informed interpretation of the analytical results. That is, unexpected analytical results may be due to intrinsic properties of the nucleic acids such as the nucleic acid quality and quantity and/or extrinsic properties of the extract such as the presence of inhibitors. Interpretation of the analytical results is facilitated with the knowledge of the nucleic acid quality and quantity.

This account is divided into the following sub-sections:

1. ***Non nucleic acid-based quantification methods***
    a. Macroscopic and Microscopic examination
    b. Chemical and immunological methods
2. ***Total genomic methods- DNA based***
    a. Both intact and degraded
        i. UV Spectrophotometry
        ii. Pico-green homogeneous microtitre plate assays
    b. Intact vs. Degraded DNA
        i. Agarose yield gel electrophoresis
3. ***Human and higher primate specific methods – DNA based***
    a. Both intact and degraded
        i. Slot Blot Hybridization using a D17Z1 probe
        ii. AluQuant
    b. Intact vs. Degraded DNA
        i. Southern analysis of agarose yield gel-blots with D17Z1
4. ***Real-time PCR, DNA-based human target specific methods***
    a. Total human autosomal DNA
    b. Alu repeat, Y chromosome DNA, Mitochondrial DNA Real-time PCR
    c. Multiplex Real-Time PCR
    d. Intact vs. Degraded DNA
5. ***End-point PCR DNA quantification and alternate DNA detection methods***
6. ***RNA based quantification methods***
7. ***Sources of Variation in Quantification methods***
    a. DNA quantification standards
    b. Interlaboratory studies on DNA quantification

In 2003, Nicklas and Buel published an excellent detailed review of quantification of DNA in forensic samples and many of these methods are also covered in Butler (2005).

1. Non nucleic acid based quantification methods

Assessment of biological evidence will usually start with a macroscopic visual examination. Visual examination may be performed using ambient light, additional white light sources, UV lights and/or alternate light sources in conjunction with filter glasses. Both visual examination and tactile examination (using gloves) may be performed with both resulting in a crude estimate of the quantity of the amount of biological evidence. Historically, this type of visual and tactile examination was the first type of "quantification". For example, investigators were told that approximately a 'dime sized' bloodstain was required for the RFLP method.

Screening for common biological fluids such as blood, semen and saliva using chemical, immunological and/or microscopic examinations of the evidence may also be performed. Although the results of those examinations are mainly qualitative, they can also provide a crude estimate of expected quantity of DNA per unit area of an evidence sample since they estimate relative amounts of biological fluid. Limitations of using this as a quantification method are that the total number of cells that contain the DNA may not always be the same within the sample nor between samples due to differences in cell counts per volume of biological fluid, distribution of cells in the evidence or due to sampling differences. Knowledge of the range of cells per unit volume has also been invoked in estimating the amount of DNA from FTA paper.

The use of microscopic examinations on cellular material that is used as a confirmatory test of spermatozoa, is more indicative of the expected DNA yield from the evidence since the amount of DNA per cell is known. For example, microscopic examination of sexual assault evidence may reveal the presence of spermatozoa. Analysts may sample known size cutting or amount of the evidence, dilute by a known factor, place an aliquot on a microscopic slide and then count the number of cells present and estimate the amount of cells per unit of evidence. This visual examination may be used to confirm the presence of spermatozoa (and thus male DNA) and determine an estimate of the expected amount of male DNA by multiplying by the number of cells observed per unit area by the pg/cell type (6 pg for a diploid cell and 3pg for haploid cell).

2. Total genomic methods- DNA based

The first analytical method used in forensic DNA, Restriction Fragment Length Polymorphisms (RFLPs) of Variable Number of Tandem Repeat (VNTR) loci, required a relatively large amount (50ng) of intact genomic DNA. Methods developed and implemented included those that assessed quantity and quality of the genomic DNA.

Early methods that were utilized to determine total nucleic acids were UV Spectrophotometry (A260/280), and picogreen microtitre plate homogeneous assays.

The Picogreen assay could detect as little as 25pg/ml (Singer et al. 1997). The Pico Green dye is an intercalating dye and in the presence of DNA displays enhanced

fluorescence. However, need to assess quality required an assessment of the amount of resulting DNA that was intact versus degraded. In many forensic DNA laboratories, this assessment was accomplished using agarose yield gel electrophoresis of whole genomic DNA with quantification standards. DNA in the gels was visualized using a fluorescent dye such as ethidium bromide and then the gel was photographed with a UV light and appropriate filter.

3. Human and higher primate specific methods – DNA based

Since biological evidence may contain non-human sources, determining the amount of human DNA in the evidence is needed. In order to assess the total amount of human/higher primate DNA, the first method utilized was a southern hybridization of a human/higher primate specific probe, D17Z1 to extracted DNA bound to nylon membranes (Waye et al. 1989, Walsh et al. 1992). In forensic DNA laboratories, the method was called a Slot blot. Detection is performed using a colorimetric or chemilluminescent enzyme linked assay and can be performed using a CCD camera (Budowle et al. 2001).

Quantification of the DNA was required not only to determine the amount to utilize for the digestion of the DNA in RFLP, but also to load approximately the same amounts of restricted genomic DNA onto a single gel. This would result in hybridization of probes to target amounts that were as close as possible in amounts. In this way autoradiographs would contain approximately equivalent signals (band patterns) for every sample and every probe.

Other methods have been developed that quantify total human DNA. These include a human quantification system called the AluQuant (Mandrekar et al. 2001). AluQuant assay targets the Alu repeats that are in high copy number in the human and primate lineages. In this assay the hybridization starts a cascade of enzymatic reactions ending in the production of light read by a luminometer. The assay has a dynamic range from 0.1 to 50ng. Another method that was utilized to determine the amount of human intact vs. degraded DNA was a simple southern analysis of the yield gel using a D17Z1 probe that was called a yield gel blot.

4. Real-time PCR, DNA-based human target specific methods

Current methods using the polymerase chain reaction (PCR) to amplify autosomal short tandem repeats and other loci require an optimal range of input DNA amounts with either too much or too little resulting in non-optimal amplification. Therefore, the most sensitive method with the widest dynamic range is desirable. Real-time QPCR using a 5′-nuclease fluorogenic or TaqMan assays can be used to determine the starting amounts of human DNA.

Real-time QPCR has several advantages over the other methods in that it is extremely accurate and sensitive over a broad dynamic range, and it occurs in a closed-tube system, reducing the potential for carryover contamination. Using this technique, a forensic biologist can monitor and quantify the accumulation of PCR products during log phase amplification. (Heid et al., 1996).

Several RT PCR human specific assays are now available that target autosomal, Alu repeats, Y chromosome and mtDNA (Andréasson et al. 2002, von Wurmb-Schwark et al. 2002, Nicklas and Buel 2003, Alonso et al. 2004, Nicklas and Buel 2005, Green et al. 2005, Andréasson et al. 2006, Horsman et al. 2006, Westring et al. 2007). The assays may be performed on single targets or in multiplexes (Timken et al. 2005, Walker et al. 2005, Nicklas et al. 2006). RT PCR assays have been utilized to detect degraded vs. intact human DNA and PCR inhibitors (Swango et al. 2006). In addition, a multiplex quantitative PCR assay that amplifies two human nuclear DNA target sequences of different length to assess DNA degradation and a third amplification target, a synthetic oligonucleotide internal PCR control (IPC), to allow for the assessment of PCR inhibition has been reported (Swango et al. 2007). The use of qPCR to detect inhibitors have been also reported by Kontanis and Reed (2006) and Davorin et al. (2007).

Targets that have been used for human DNA quantification kits include the human telomerase reverse transcriptase (hTERT) locus located on chromosome 5 (Quantifiler assay Green et al. 2005), the "sex reversal" (SRY) locus located on the Y chromosome (Quantifiler Y assay- Green et al. 2005), and Alu repeats (H-quant - Shewale et al. 2007). Another report utilized amelogenin as a target for human quantification (Allen and Fuller 2006).

5. End-point PCR DNA quantification and alternate DNA detection methods

Quantification of human targets such as a single STR locus or an Alu repeat is amplified with known amounts of DNA standards. A standard curve is generated and the unknowns' concentrations can be calculated against the curve (Fox et al. 2003, Nicklas and Buel 2003).

Alternative DNA detection methods utilize fluorescent (Alivisatos et al. 1996) and electrochemical (Bardea, et al. 1999, Park, et al. 2002, Patolsky et al.2002) methods. In addition, alternate optical detection methods are based on hybridization between target DNA and substrate modified with radioactive, fluorescent, chemiluminescent or nanoparticle tags (Maxwell et al. 2002, Li and Rothberg 2004, Gill et al. 2005). The use of gold nanoparticles (nAu) as labeling tags have resulted in highly sensitive detection assays (Dubertret et al. 2001, Mawell et al. 2002, Qin and Yung 2007) and can reach attomolar and high zeptomolar sensitivity (Nam et al. 2004, Taton et al. 2000, Thaxton et al. 2005). Such sensitivity might permit direct detection of genomic DNA and according to the authors, may bypass the need of PCR amplification (Qin and Yung 2007).

6. RNA based quantification methods

Different genetic expression patterns (mRNAs) exist in different tissue types. Body fluid identification has been reported based on their mRNA profiles (Juusola and Ballantyne 2003, 2005, and 2007, Nussbaumer et al. 2006) In addition, the age of a bloodstain was reported using analysis of mRNA: rRNA ratios (Anderson et al. 2005). This information may be useful in establishing the time of the crime.

Advantages of the mRNA-based approach, versus the conventional biochemical tests, include greater specificity, simultaneous and semi-automatic analysis, rapid detection, decreased sample consumption and compatibility with DNA extraction methodologies. The quantification of the amounts of the mRNA species relative to housekeeping genes is a critical aspect of the assays (Juusola and Ballantyne 2003).

7. Sources of Variation in Quantification methods

Quantification methods whether used in the past or present are estimates as the accuracy and precision of the methods may be affected by several factors. Among these are the stability of the quantification standards, pipetting variability and kit reagent stability. In addition, variation in the storage conditions and tube types as well as the extraction method used may result in variability.

A series of interlaboratory studies has been conducted by the US National Institute of Standards and Technology on quantitation (Duewer et al. 2001, Kline et al. 2003 and 2005). In one study conducted by the US National Institute of Standards and Technology (Kline et al. 2003), a ten-fold range of reported concentrations was observed for the same sample among different laboratories. Variation was attributed to several sources including differences in pipetting, quantification method used, DNA sample stability, and variation in quantification standards. Estimates of the quantities of DNA on the 'same' sample may vary between laboratories using the same methods (Kline et al. 2005) and this has spurred the development of a human DNA quantitation standard, SRM 2372[1].

[1] http://www.cstl.nist.gov/div831/strbase/pub_pres/Kline_NIJ2006.pdf

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