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Development and validation of InnoQuant™, a sensitive human DNA quantitation and degradation assessment method for forensic samples using high copy number mobile elements *Alu* and SVA

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ABSTRACT

There is a constant need in forensic casework laboratories for an improved way to increase the first-pass success rate of forensic samples. The recent advances in mini STR analysis, SNP, and *Alu* marker systems have now made it possible to analyze highly compromised samples, yet few tools are available that can simultaneously provide an assessment of quantity, inhibition, and degradation in a sample prior to genotyping. Currently there are several different approaches used for fluorescence-based quantification assays which provide a measure of quantity and inhibition. However, a system which can also assess the extent of degradation in a forensic sample will be a useful tool for DNA analysts. Possessing this information prior to genotyping will allow an analyst to more informatively make downstream decisions for the successful typing of a forensic sample without unnecessarily consuming DNA extract. Real-time PCR provides a reliable method for determining the amount and quality of amplifiable DNA in a biological sample.

Alu are Short Interspersed Elements (SINE), approximately 300 bp insertions which are distributed throughout the human genome in large copy number. The use of an internal primer to amplify a segment of an *Alu* element allows for human specificity as well as high sensitivity when compared to a single copy target. The advantage of an *Alu* system is the presence of a large number (>1000) of fixed insertions in every human genome, which minimizes the individual specific variation possible when using a multi-copy target quantification system. This study utilizes two independent retrotransposon genomic targets to obtain quantification of an 80 bp "short" DNA fragment and a 207 bp "long" DNA fragment in a degraded DNA sample in the multiplex system InnoQuant™. The ratio of the two quantitation values provides a "Degradation Index", or a qualitative measure of a sample's extent of degradation. The Degradation Index was found to be predictive of the observed loss of STR markers and alleles as degradation increases. Use of a synthetic target as an internal positive control (IPC) provides an additional assessment for the presence of PCR inhibitors in the test sample.

In conclusion, a DNA based qualitative/quantitative/inhibition assessment system that accurately predicts the status of a biological sample, will be a valuable tool for deciding which DNA test kit to utilize and how much target DNA to use, when processing compromised forensic samples for DNA testing.

1. Introduction

In recent years, real-time PCR has become the standard for quantifying the amount of genomic DNA in a forensic sample. Commonly used systems include the assessment of total human and male DNA [1–3]. Forensic samples vary widely in quantity and quality. The aim of this study is to develop and validate a real-time PCR system to assess both the quality and quantity of DNA in a sample. The system is designed to detect total human DNA, and to qualitatively measure the extent of degradation by using two nuclear DNA targets: a multi-copy sequence of "short" length [4] and a separate multi-copy sequence of "long" length [5]. The quantity ratio of the short target to the long target will provide an assessment of the extent of degradation in the sample, and therefore provide information that can be used to select the best

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typing system, thereby improving sample success rate and eliminating unnecessary rework. Previous studies on the assessment of degraded DNA in a forensic sample have been published using *Alu* [6] or mini-satellite [7] targets. However, these assays lack reproducibility and/or sensitivity, or do not exhibit high PCR efficiencies. In the case of a previous study using *Alu* elements [6], the authors conclude the low PCR efficiencies are because the two amplicons are competing. This is most likely due to the fact that the two Ya5 *Alu* element fragment sequences targeted were not independent. The present study utilizes two multi-copy, independent targets: Yb8 *Alu* element and SVA element for the two different sized targets for human DNA quantitation. Although use of Yb8 *Alu* element amplification for DNA quantitation has been previously reported [4,8,9], this is the first report of the use of intra SVA element for DNA quantitation.

Alu elements are transposable elements which have amplified to a copy number of over 1 million elements throughout primate evolution and comprise roughly 10% of the human genome [10]. This amplification produced a series of subfamilies of *Alu* elements that appear to be of different genetic ages. The expansion of these elements throughout primate evolution has created several recently integrated "young" *Alu* subfamilies that are present in the human genome but are largely absent from non-human primates [10]. These human-specific subfamilies only have a fraction of the copy number compared to primate-specific elements, but when approached collectively, human-specific and primate-specific *Alu* based assays provide a powerful tool for sensitive human DNA identification and quantitation. Because of their high copy number, *Alu* elements are a naturally amplified source of human genetic information. While some recently integrated *Alu* insertions remain polymorphic in the human population, many ultimately reach fixation for the presence of the *Alu* insertion. The two retrotransposon elements examined in the present study are Yb8 and SVA. The Yb lineage is the second largest "young" group of evolutionarily related *Alu* subfamilies in the human genome [11]. In addition to the major retrotransposon families, there are smaller families of transposons such as SVA [5]. SVA elements, named after its main components, SINE, VNTR and *Alu*, contain the hallmarks of retrotransposons, in that they are flanked by target site duplications (TSDs), terminate in a poly(A) tail and they are occasionally truncated and inverted during their integration into the genome [5].

Alu element based quantitation methods are advantageous when compared to current, commercially available systems. The advantage of an *Alu* system is the presence of a large number of fixed insertions. Due to the high copy number of subfamily-specific *Alu* repeats within the human genome, these human-specific DNA assays have a very sensitive dynamic range of 0.001–100 ng [12]. The use of *Alu* sequence based, real-time PCR has been reported in scientific literature as a more sensitive method for the quantitation of genomic DNA compared to other approaches currently used in most forensic laboratories [4,8,13–15]. Nicklas and Buel [6,13] have targeted the Ya5 subfamily of *Alu* elements for quantitation of human DNA. Walker et al. [8] have reported an assay for simultaneous quantitation of human autosomal, male and mitochondrial DNA using the *Alu* elements Yb8, Yd6, *Alu*STXa and *Alu*STYa. These scientific findings support the assertion that *Alu* based assays have superior sensitivity because of their high copy number and can be successfully used for quantitation of human DNA in biological samples in forensic casework. Additionally, the Yb *Alu* lineage contains approximately 1800 copies per genome [11] and SVA contains approximately 1700 full length element copies per genome [5,16]. This large copy number minimizes the effect of variation between individuals, resulting in highly reproducible quantitation values.

The present study uses two independent retrotransposable element genomic targets, an *Alu* element in the Yb8 subfamily [4] and an SVA element [5], in a multiplex based, real-time PCR assay for the detection of two sized targets to assess the extent of degradation in a forensic sample. The system is highly sensitive due to its multi-copy nature, and highly reproducible and accurate due to its high copy number (>1000 copies per genome).

2. Materials and methods

2.1. Standards and samples

DNA extracted from in-house reference buccal and/or blood samples, was used in all studies, as well as DNA extracted from semen, hair and teeth samples for the non-probative evidence sample study. All reference and casework type samples were extracted in-house using the ChargeSwitch® magnetic bead extraction [17] or organic (ProK/SDS digestion, phenol/chloroform extraction and Microcon concentration into Tris–EDTA buffer, TE^{-4}, 10 mM Tris–0.1 mM EDTA, pH 8.0) [18] extraction methods. For the tooth extractions, a single tooth was ground into a fine powder and extracted using the organic extraction method as described. Sperm samples were lysed by a ProK/SDS/DTT digestion and extracted using ChargeSwitch® magnetic bead extraction (Invitrogen). Shaft portions of the hair samples were dissected into 2-centimeter length fragments, and extracted using an organic extraction method [18] as described with a modified lysis step. High molecular weight genomic male DNA was used to create the standard curve for each validation experiment. High molecular weight DNA (either single source or mixture) was purchased from EMD Millipore or Amsbio (Lake Forest, CA). DNA suspension buffer (TE^{-4}, 10 mM Tris–0.1 mM EDTA) was purchased from Teknova, Hollister, CA. NIST SRM 2372 (Human DNA Quantitation Standard) was used as a known quantitation standard to assess the accuracy of the system [19].

2.2. Selection of target sequences and characterization of genetic markers

The aim of this study was to develop a sensitive, real-time PCR quantitation assay to assess degradation in forensic DNA samples. A critical element of this study was to develop a very sensitive assay, given the low template nature of forensic samples. For this reason, multi-copy elements were chosen for the study. Initially, two REs were studied: a ~80 bp fragment in the Yb8 *Alu* subfamily and a ~250 bp fragment in the *Alu* Ya5 subfamily [20]. Experimentation with a real time PCR multiplex composed of Yb8 and Ya5 targets worked well; however, cross reactivity was observed due to sequence similarities between these two targets. Because of these sequence similarities, it was observed that the primers could cross amplify the targets sequences, thereby yielding less than accurate results for one or the other target sequence.

To make the two target sequence amplifications independent of each other, two retrotransposons, *Alu* Yb8 [4,21] and SVA [5] were selected as the targets for amplification of the short and long sequences, respectively. An intra RE primer design was used to target an 80 bpYb8 *Alu* sequence for the "short" fragment as well as a 207 bp sequence in the SINE-R region of SVA for the "long" fragment. To assess the presence or absence of inhibitors in the sample, a synthetic nucleotide sequence used as an internal positive control (IPC) was also evaluated.

The quantitation real time PCR multiplex reported here is a three target system containing the Yb8 "short" *Alu* fragment labeled in FAM, the SVA "long" fragment labeled in Cy5 (Fig. 1), and the IPC labeled in Cy3. The primers were selected and designed