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Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA

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ABSTRACT

Multiplex human short tandem repeat analysis demands reliable DNA quantification to consistently produce interpretable genotypes. The Plexor® HY System is a multiplex quantitative PCR assay to quantify total human and male DNA. We performed developmental validation of the Plexor® HY System to demonstrate the performance capabilities and limitations of the assay for forensic applications. Validation studies examined: (a) human specificity, (b) sensitivity, (c) quantification of degraded DNA, (d) impact of inhibitors, (e) male/female mixture and Y-assay male specificity, (f) reproducibility and concordance and (g) population studies.

1. Introduction

Multiplex short tandem repeat (STR) analysis is the core technology in DNA-based human identification. These assays require a defined range of template quantities to produce optimal results. In addition to accurate sample quantification, assessment of sample quality and sensitive detection are necessary to determine how best to proceed with sample analysis.

Quantitative PCR (qPCR) has displaced hybridization-based methods for human-specific DNA quantification in forensic applications. qPCR has reduced the rate of false-negative STR results due to lack of sensitivity and increased the objectivity of data interpretation by providing a numerical output rather than requiring a visual comparison of band intensities. However, some current qPCR methods do not allow simultaneous quantification of total human and male DNA or do not have a level of sensitivity that consistently exceeds that of subsequent STR assays.

The Plexor® HY System is a qPCR assay that simultaneously quantifies total human DNA and male DNA [1–3] using the Plexor® technology, which results in decreasing fluorescence as the amplification progresses [4–7]. The triplex configuration allows co-amplification of a human autosomal sequence, a human Y-chromosomal sequence and a novel exogenous control sequence to quantify total human DNA and male DNA and provide an internal PCR control (IPC), respectively.

The autosomal primers are labeled with fluorescein and amplify a 99 bp sequence from the human RNU2 locus. The RNU2 locus encodes a small nuclear RNA involved in pre-mRNA processing. This region is conserved among primates and organized as a tandemly repeated motif (~6 kb each) on the long arm of chromosome 17 [8–15]. The Y-chromosome primers are labeled with CAL Fluor® Orange 560 (Biosearch Technologies, Inc.) and target a 133 bp sequence from the testis-specific protein, Y-encoded (TSPY) locus. The TSPY gene is involved in spermatogenesis and is conserved in primates [15–18]. The TSPY locus is within the DYZ5 region, a 20 kb repeated motif on the Y chromosome. The IPC primers are labeled with CAL Fluor® Red 610 (Biosearch Technologies, Inc.) and detect a novel IPC sequence, which is included as a template in all reactions. The amplified IPC product is 150 bp. Data from the IPC amplifications are used to monitor amplification inhibition. A fourth dye, IC5, is included in all wells and used as a passive reference. Data from the three amplifications can be normalized to the passive reference signal to reduce the impact of instrument-specific signal fluctuation.

The findings presented here document the basic performance characteristics of the Plexor® HY System as part of a manufac-

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turer's validation. Given these results, laboratories implementing the Plexor® HY System may consider omitting some of these studies from their internal validation as previously described by the manufacturer [19].

2. Materials and methods

2.1. DNA

The Plexor® HY Male Genomic DNA Standard, provided with the Plexor® HY System, was used to generate all standard curves. This DNA is a mixture of several human male DNAs and is not derived from cell lines. Except where noted, we generated standard curves by amplifying a fivefold serial dilution of the DNA standard from 16 pg/μl to 50 ng/μl. We purified male and female human DNA for use as unknown samples from liquid blood using organic extraction [20] or from buccal swabs. Buccal swabs were heated to 90 °C for 30 min in 400 μl of SV Lysis Buffer (Cat.# Z3052) containing 10 mM DTT. Samples were centrifuged through a DNA IQ™ Spin Basket (Cat.# V1221) for 2 min at 14,000 × *g* in a microcentrifuge to collect the lysate. DNA was purified using the Wizard® SV Genomic System (Cat.# A2361) and the genomic DNA purification protocol from lysates using a microcentrifuge [21]. Samples were eluted in 100 μl of TE–4 buffer [10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA].

Pig, mouse, rabbit, macaque, Japanese macaque, ferret, cheetah, reindeer, elephant, cat, warthog, straw-colored fruit bat, wallaby, mandrill, tamarin, red panda, rat, rhesus monkey, orangutan, hyena and *Saccharomyces cerevisiae* DNA samples were kindly provided by Roger Frappier of the Centre of Forensic Sciences. DNA from calf thymus (bovine), *Escherichia coli* (strain B), *Micrococcus lysodeikticus* and *Clostridium perfringens* was obtained from Sigma–Aldrich. Dog, chicken, rabbit, rat, human papilloma virus, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and hepatitis B virus DNA were kindly provided by Cecilia Crouse of the Palm Beach County Sheriff's Office. An isolate of *Candida albicans* was kindly provided by Dr. Kenneth W. Nickerson (University of Nebraska—Lincoln). Herring sperm DNA (Cat.# D1811) was acquired from Promega. In general these DNA samples were purified using organic extraction and quantified by spectrophotometric analysis using A260 detection [20] or comparison to DNA standards on an ethidium bromide-stained agarose gel [20].

2.2. Real-time PCR cycling and detection

Unless noted, all samples were analyzed in duplicate, and the average results are reported. All testing of the Plexor® HY System was performed using the Applied Biosystems 7500 Real-Time PCR System. Where noted, additional testing was performed using an Applied Biosystems 7500 FAST Real-Time PCR System (in standard 7500 mode) or Stratagene Mx3005P® Quantitative PCR System. Cycling parameters were: 1 cycle at 95 °C for 2 min; 38 cycles of 95 °C for 5 s, then 60 °C for 35 s (7500) or 40 s (Mx3005P®) with data collection during the elongation/extension step. Post-amplification melt analysis was performed using the default dissociation analysis (7500) or a 48-step protocol that increased the temperature 0.6 °C per step from 65 to 92 °C and had two data collection points per step (Mx3005P®). Instrument calibration (7500 only), setup and programming were performed as described in the Plexor® HY System Technical Manuals [2,3].

For concordance studies, the Quantifiler® human and human male DNA quantification kits (Applied Biosystems) were used as directed by the manufacturer.

2.3. Plexor® data analysis

The Plexor® Analysis Software interprets amplification data with decreasing fluorescence, generates standard curves and calculates DNA concentrations of unknowns. We used the forensic release of the Plexor® Analysis Software, version 1.5.4.10, to analyze all data reported. Following data collection on each instrument, raw data was exported from the instrument software, then imported into the Plexor® Analysis Software [2,3]. The Plexor® Analysis Software generated the amplification curve, cycle threshold (C_T), melt curve, melt threshold, product melt temperature (T_m), standard curve and unknown sample quantification data. Following quantification of unknowns, we used an STR normalization module to (a) compute sample input volumes required for autosomal STR and Y-STR amplifications, (b) calculate necessary dilutions for concentrated DNA samples and (c) flag low-quality data and inhibited quantification reactions.

2.4. Inhibitors

We examined the effect of three inhibitors: hematin, humic acid and calcium chloride. These inhibitors were added to samples prior to quantification and subsequent STR analysis. Porcine hematin (Sigma–Aldrich) was resuspended to 1 mM in 1N sodium hydroxide. Humic acid (Sigma–Aldrich) was resuspended at 0.5 μg/μl in water. Calcium chloride (Promega) was prepared at 100 mM in water.

2.5. STR analysis

We performed STR analysis to correlate changes in quantification results and the impact on genotyping. In general, peak height (yield) and the number of observed alleles were the primary considerations when interpreting STR data. We used autosomal quantification data ([AUTO]) to normalize DNA input into PowerPlex® 16 reactions (Cat.# DC6530) and Y-chromosomal quantification results ([Y]) to normalize DNA input into PowerPlex® Y System (Cat.# DC6760) reactions. In general, we amplified 0.5 ng of DNA in a 25 μl STR reaction using 32 cycles as described in the PowerPlex® 16 and PowerPlex® Y Systems Technical Manuals. After amplification, we prepared samples by combining 1 μl of sample, 9.5 μl of Hi-Di™ formamide (Applied Biosystems) and 0.5 μl of Internal Lane Standard 600 (ILS600, Cat.# DG2611). Separation and detection of STR amplification products were performed using an Applied Biosystems 3130 or 3130xl Genetic Analyzer using a 10-s, 3 kV injection or 5-s, 3 kV injection, respectively. Data analysis was performed using the GeneMapper® ID software. We limited interpretation of electropherograms to peaks above a minimum detection threshold of 50 relative fluorescence units (RFU).

See supplemental report at www.fsigenetics.com for materials and methods of degradation studies.

3. Results and discussion

3.1. Species specificity

DNA purified from forensic samples commonly contains a mixture of human DNA and contaminating DNA from bacteria, fungi, viruses or other organisms, and some samples may not include any human biological material. Consequently, STR genotyping assays for forensic use are generally reactive to human and primate DNA only [22,23]. Similarly, the quantification system must not react to non-primate DNA.