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Developmental validation of the EX20+4 system

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ABSTRACT

The EX20+4Y System is a polymerase chain reaction (PCR)-based amplification kit that enables typing of 19 autosomal short tandem repeat (STR) loci (i.e., CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX, vWA, Penta D, Penta E, D2S1338, D19S433, D12S391, D6S1043), four widely used Y chromosome-specific STR (Y-STR) loci (DYS458, DYS456, DYS391, DYS635), and amelogenin. In this study, this multiplex system was validated for sensitivity of detection, DNA mixtures, inhibitor tolerance, species specificity based on the Scientific Working Group on DNA Analysis methods (SWGDAM) developmental validation guidelines, and the Chinese criteria for the human fluorescent STR multiplex PCR reagent. The results show that the EX20+4 System is a robust and reliable amplification kit which can be used for human identification testing.

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1. Introduction

The rapid growth of DNA databases require more markers to be included in multiplex kits to meet the challenges of adventitious hits, missing person identification, international data sharing, etc. [1,2]. With the current short tandem repeat (STR) and capillary electrophoresis (CE) technology, which can multiplex up to about 20–25 STR loci, a combination of autosomal STR loci and several Y chromosome-specific STR (Y-STR) loci can provide a higher kinship index with sufficient discriminating power for single-source comparisons compared with a similar number of only autosomal STR loci [2]. With a nominal number of Y-STR loci, gender of the sample donor can be determined with high accuracy, even with amelogenin-Y dropout [1,3]. In samples where there is a much larger contribution of female DNA compared with male DNA, Y-STR typing can be beneficial [4].

In this study, we developed and validated the EX20+4 System. This multiplex is a 5-dye system that enables typing of 19 autosomal STR loci, four Y-STR loci, and amelogenin. The blue channel consists of fluorescein-labeled STR amplicons for the D3S1358, D13S317, D7S820, D16S539, Penta E, and DYS635 loci. The green channel includes DNA fragments labeled with hexachlorofluorescein (HEX) for the DYS456, TPOX, TH01, D2S1338, CSF1PO, Penta D, and DYS458 loci. The yellow channel consists of amplification products for the DYS391, D19S433, vWA, D21S11, D18S51, and D6S1043 loci that are labeled with tetramethylrhodamine (TAMRA). The amplified products for amelogenin, D8S1179, D5S818, D12S391, and FGA loci are in the red channel and labeled with 6-carboxy-X-rhodamine (ROX). Fragments included in the internal lane standard are detected in the orange channel and are labeled with SIZ-500.

Scientific Working Group on DNA Analysis methods (SWGDAM) developmental validation guidelines [5] and the Chinese criteria [6] for human fluorescent STR multiplex polymerase chain reaction (PCR) reagent are followed in the development and validation of the EX20+4 System.

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2. Materials and methods

2.1. Samples

For the sensitivity study, male 2800M and male 9948 human genomic DNA (Promega, Madison, WI, USA) were amplified in a serial dilution with the following template amounts: 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, and 31.25 pg.

Species specificity studies were conducted using 1 ng each of purified DNA from human, chimpanzee, and macaque; 10 ng each of dog, pig, cat, sheep, chicken, mouse, rabbit, fish, and a microbial pool (1 ng each purified DNA from *Escherichia coli*, *Lactobacillus acidophilus*, *Salmonella*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus salivarius*, *Corynebacterium pyogenes*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus flavus* mixed together); and a negative control. These samples were kindly donated by the Guangzhou Zoo (Guangzhou, China) and the Institute of Human Virology of Sun Yat-Sen University (Guangzhou, China). The quantity was determined by Qubit® ssDNA Assay Kit with the Qubit® 2.0 Fluorometer (Invitrogen/Life Technologies, San Francisco, CA, USA).

Several common forensic inhibitors were tested including hematin, indigo, humic acid, calcium ion, hemoglobin, and ethylenediaminetetraacetic acid (EDTA). The male DNA template quantity was held constant at 500 pg, while the concentration of inhibitors was varied: 0, 120, 160, or 200 μmol/L of hematin; 0, 500, 750, or 1000 μmol/L of indigo; 0, 5, 10, or 20 ng/μL of humic acid; 0, 1.0, 1.5, or 1.75 mmol/L of calcium ion; 0, 500, 750, or 1000 μmol/L of hemoglobin; and 0, 0.8, 1.0, or 1.2 mM of EDTA.

Male/male mixtures were prepared using male 9948 and male 2800 M human genomic DNA (Promega) with mixture ratios of 1:1, 1:4, 1:9, or 1:19. Male/female mixtures were prepared using male 9948 and female 9947a human genomic DNA with mixture ratios of 1:1, 1:4, 1:9, or 1:19. Each mixture was tested in triplicate. These samples were formulated at a concentration of 1 ng in 10 μL. An additional male/female mixture was set up using a constant template of 500 pg male DNA 2800 M with an increasing amount of female DNA: 50 ng, 100 ng, 200 ng, 400 ng, and 800 ng.

Reproducibility samples included buccal-indicating FTA® Cards, blood FTA® Cards (GE Healthcare/Whatman, Buckinghamshire, UK), and extracted DNA from one female donor and two male donors. The DNA was extracted by magnetic beads (DNA IQ™ System, Promega, Madision, WI, USA) on the Maxwell® 16 Research System (Promega). The quantity was determined by the Quantifiler Human DNA Quantification Kit with the 7500 Real-time PCR System (Life Technologies). Three laboratories (i.e., Guangzhou Forensic Science Institute and two of its sub-institutes) participated in the tests.

Case samples were tested at the Guangzhou Forensic Science Institute. These samples include three bloodstains, three bones, three epithelial fractions, and three buccal-indicating FTA® Cards. Buccal-indicating FTA® Cards were amplified directly while others were extracted by magnetic beads. The results were compared with a PowerPlex21 kit (Promega, Madision, WI, USA).

Stutter calculation studies contain 130 extracted DNA samples which were amplified and analyzed on a 3130xl Genetic Analyzer (AB, Foster City, California, USA). These samples were collected with informed consent from 130 donors of the Guangdong Han population. These samples were extracted by magnetic beads from cotton buccal swabs on the TECAN® Freedom EVO workstation (TECAN, Männedorf, Switzerland). Each amplification reaction contained 0.5 ng DNA in a 25-μL volume. The quantity was determined by the Quantifiler Human DNA Quantification Kit with the 7500 Real-time PCR System (Life Technologies).

Population studies were performed with samples from 500 unrelated individuals (250 males and 250 females) of the Guangdong Han population that were collected with informed consent. The blood samples were maintained on blood FTA® Cards (GE Healthcare/Whatman). Concordance testing was performed with 200 blood FTA® Card (GE Healthcare/Whatman) samples, including 100 males and 100 females. These samples were a subset of the 500 population study samples.

2.2. DNA amplification

Amplifications were performed on a GeneAmp® PCR System 9700 thermal cycler (Life Technologies) using the 9600 mode on a gold-plated silver block. Each amplification reaction contained 10 μL of reaction mix, 5 μL of EX20+4 primers, and 1 μL of C-Taq DNA polymerase (AGCU ScienTch Incorporation, Wuxi, China) with up to 9 μL of template. According to the manufacturer's technical manual, unless specifically mentioned otherwise, the following amplification setup and cycling parameters were used to amplify extracted DNA: initial denaturation of 95 °C for 2 min; 10 cycles of 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 1 min; 20 cycles of 90 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; a final extension at 60 °C for 60 min; and a 4 °C soak. Samples used for direct amplification were processed using the same cycling parameters with the exception of the cycle number 29 (the cycle number of the second step was reduced to 19). Direct amplification reactions were performed using one 1.2-mm punch from blood FTA® Cards and two punches from buccal-indicating FTA® Cards. Negative controls were included in all experiments.

2.3. Electrophoresis and analysis

Spectral resolution was established using the AGCU 5-dye Matrix Standards to assess each fluorescent dye contained in the kit. Reactions were prepared for CE by combining 12.0 μL of Hi-Di™ Formamide (AB, Foster City, California, USA), 0.5 μL of AGCU Marker SIZ-500, and 1.0 μL of the sample or allelic ladder. Samples were denatured for 3 min at 95 °C, then immediately quenched on ice. Electrophoresis was performed on the 3130xl (16-capillary) Genetic Analyzer using 36-cm capillary arrays with POP-4® Polymer (AB, Foster City, California, USA). Standard run conditions were: sample injection for 5 s at 3 kV and electrophoresis at 15 kV for 1500 s with the indicated run temperature of 60 °C. GeneMapper® ID v.3.2 (AB, Foster City, California, USA) was used to determine fragment size. A peak amplitude of 50 relative fluorescence units (RFUs) was implemented as the peak detection threshold when analyzing data from CE instruments.

2.4. Statistical analysis

Stutter ratios were calculated as stutter peak height divided by the peak height of the associated allele.

Population statistical analysis such as heterozygosity (H), discrimination power (DP), polymorphism information content (PIC), and power of exclusion (PE) were calculated using Power Stats v1.2 software (Promega, Madision, WI, USA). Total discrimination power (TDP) and cumulative probability of paternity exclusion (CPE) were calculated according to Ref. [6].

The fluorescence balance of heterozygous alleles (intra-locus balance) was calculated as lower peak height divided by higher peak height at one locus. The balance within the same fluorescent dye (intra-color balance) was the peak height ratio between the minimum peak height (average height at one locus) and the maximum height (average height at one locus) in the same group. The balance across all loci (inter-color balance) was calculated in a similar way as the intra-color balance.

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