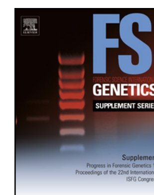
Forensic Science International: Genetics Supplement Series 5 (2015) e656–e658

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

journal homepage: www.elsevier.com/locate/FSIGSS



Easy and fast procedure to isolate, purify and immortalize DNA fragments for allelic ladders construction

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ARTICLE INFO

Article history:
Received 30 August 2015
Accepted 8 October 2015
Available online 30 October 2015

Keywords:
Allelic ladder
Allele isolation
NC01
Ladder construction

ABSTRACT

Allelic ladders are essential quality standards in forensic casework analysis with STRs. These are obtained by mixing individual genotypes, however, when alleles are obtained from heterozygous individuals, they cause allelic unbalance in the ladder mix and dependence upon original DNA extracts availability. In this work alleles were amplified and separated from heterozygous state by agarose or polyacrylamide gels after electrophoresis. Target alleles were recovered by gel puncturing and then reamplified; subsequent dilutions (10^{-3}–10^{-4}) of these amplificates were used as new templates for PCR with fluorolabelled primers. This strategy was successfully used to obtain balanced allelic ladders for mini STRs D10S1248 and D14S1434 tetra nucleotides which are part of NC01 (Non Codis) triplex. It is also worth noticing that this procedure could also be used to isolate new or mutant alleles that are to be sequenced.
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1. Introduction

Validation of human STRs requires allelic ladder construction including sequencing of some ladders alleles; quality standards for routine work also demands their use [1]. Alleles are obtained from individual genotypes, in some instances it is possible to get some alleles from homozygous individuals, but for rare, extreme and less frequent alleles it will be common to find them in heterozygous state. When building allelic ladders by direct mixing of amplificates, the use of heterozygous genotypes (which are unbalanced by definition) will cause allelic unbalance in the ladder mix and dependence upon original DNA extract availability. Separation of STRs heterozygous alleles is achieved by electrophoresis in agarose or acrylamide, then cutting individual bands and finally recovering the DNA by elution using commercial kits which are time consuming and expensive [2]; to overcome these difficulties we intend to: (1) Separate alleles in heterozygous state, by agarose electrophoresis prior to gel puncturing. (2) Reamplify this punctured DNA template under standard PCR conditions, which will render enormous amount of DNA template (allelic immortalization). (3) Mixing and balancing DNA template to amplify it and get the ladder for capillary electrophoresis. This strategy was successfully used to obtain balanced allelic ladders for D14S1434 and D10S1248 tetra nucleotide mini STRs which are part of NC01 triplex system used for analysis of degraded DNA [3–5].

2. Materials and methods

DNA samples covering full allelic range were extracted from blood stains in FTA® cards by direct Chelex method [6], including as many homozygous individuals as possible; for heterozygous individuals at least 3 repeat units apart were selected (Table S1). PCR (12,5 μl) was performed using 1,3 μM sequencing primers [3], 1U AmpliTaq Gold DNA Polymerase, 1X AmpFℓSTR® Identifiler® PCR Buffer (Appl. Biosys.), and 10 ng of DNA extract. Samples were amplified by holding temperatures up to 95 by 10 min; then cycling, 10 times at 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, 26 cycles °C for 1 min, 57 °C for 1 min, 72 °C for 1 min; additional 45 min extension at 60 °C. Amplificates were loaded into 4% agarose gels, run at low voltage (4 V/cm) and visualized by ethidium bromide staining (Fig. S1B). For allele isolation by puncturing, once agarose gel was visualized, each band was punctured (Fig. S1A) using a 10 μl pipette tip and then washed into a PCR mix previously prepared as indicated above. Amplification reaction was carried as indicated previously but with 32 amplification cycles. Once punctured, reamplified and checked

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http://dx.doi.org/10.1016/j.fsigss.2015.10.014
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for allele isolation by agarose electrophoresis (Fig. S1C), amplificates were diluted up to 500 μl and purified using microcon-100 system (Millipore). Aliquots of purified alleles were diluted 1:1.000 and 1:10.000 to produce immortalized DNA template for individual alleles. In order to generate fluorescent ladder alleles, immortalized DNA templates were amplified using genotyping fluorescent labelled primers (Fig. S2) as referred in Coble and Butler [3]. PCR conditions were the same as above, except for annealing temperature (55 °C) and 20–24 cycles was used. Labelled amplificates were electrophored using an ABI 3130 Genetic Analyzer (Appl. Biosys.) and analysed using GeneMapper ID V. 3.2 Software (Appl. Biosys.). PCR performance was estimated by signal strength; since a linear relation between signal strength (Relative Fluorescent Units) and number of DNA copies was assumed, therefore individual alleles were added to ladder mix proportionally to its signal intensity, and variation of the number of PCR cycles was also used to increase or decrease allelic signal to obtain balanced ladders.

3. Results and discussion

In order to avoid contamination during heterozygous genotypes isolation by puncturing, caution must be taken to select heterozygous individuals with alleles as far apart as possible (Table S1). When heterozygous alleles are too close, band puncturing is time consuming, and both chances of contamination and UV light exposition increase. Though inhibiting effects of agarose on PCR are known [2] we did not have PCR failure due to agarose effects. Gel puncturing proved to be reliable and easy way to isolate alleles, compared with other methods. Amplification of punctured alleles is highly specific and improves PCR performance, since template DNA is made up only of allele amplicons and that competing total genomic DNA and primers artefacts are both absent (Fig. S1C). Individual fluoro labelled alleles (Fig. S2) can be mixed in order to construct desired balanced allelic ladders (Fig. 1), avoiding genomic DNA dependence and unbalanced allelic ladders due to heterozygous genotypes imbalance. In order to balance individual alleles, dilution and cycle number must be experimentally determined, with dilutions described cycle number must be 24 or less to avoid stutter preferential amplification that can overcome target allele if cycle number is higher, causing—1 repeat ladder slippage (data not shown). Allelic ladders have been successfully rebuilt from frozen stock (−20 °C) up to six years after their initial amplification.

4. Conclusion

Gel puncturing on agarose gel was a fast and easy way to isolate DNA fragments in order to obtain high template concentrations of extreme or rare alleles to find variants (i.e. requiring sequencing), generally observed in heterozygous state. Availability of individual alleles allows for a better balance in final allelic ladder mix. Care must be taken with template dilution and PCR cycle number selection to avoid artifacts preferential amplification, and therefore diminishing signal to noise ratio.

Ethical standards

Experiments comply with the current Colombian Laws.

Conflict of interest

None.

Role of funding

This work was supported by Project 621 from “División de Investigación Científica del Instituto Nacional de Medicina Legal y Ciencias Forenses Regional Bogotá” and “Laboratorio de Identificación Genética-IdentiGEN” (Instituto de Biología, Universidad de Antioquia).

Acknowledgments

Especially thanks to INMLCF, IdentiGEN and CODI Universidad de Antioquia 2013–2014.

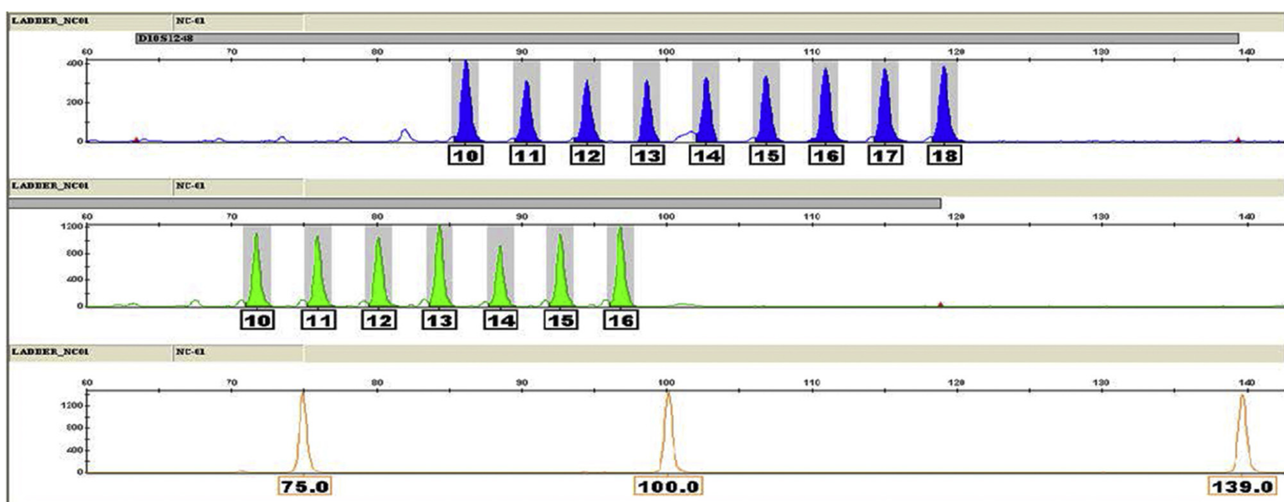

Fig. 1. Upper part: D10S1248 and D14S1434 allelic ladder electropherogram. Lower part: D10S1248 locus is shown in blue (upper), D14S1434 locus in green (middle), lower panel shows molecular weight marker Liz GS500 (Applied Biosystems). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigss.2015.10.014.

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