# Optimisation of Forensic Genetics Procedures Used in Disputed Paternity Testing: Adjustment of the PCR Reaction Volume

Damir Marjanović[1*], Narcisa Bakal[1], Lejla Kovačević[1], Melisa Hodžić, Anja Haverić[2], Sanin Haverić[2], Slavica Ibrulj[3], Adaleta Durmić[4]

1. Laboratory for Forensic Genetics, Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Kemalbegova 10, 71 000 Sarajevo, Bosnia and Herzegovina
2. Laboratory for cytogenetics and genetic toxicology, Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Kemalbegova 10, 71 000 Sarajevo, Bosnia and Herzegovina
3. Faculty of Medicine, University of Sarajevo, Čekaluša 90, 71 000 Sarajevo, Bosnia and Herzegovina
4. Laboratory for molecular genetics of natural resources, Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Kemalbegova 10, 71 000 Sarajevo, Bosnia and Herzegovina

* Corresponding author

## Abstract

Standard molecular techniques, with only a slight modification, are very useful in obtaining and interpreting the final results in the field of forensic genetic. Data obtained through such analysis are highly reliable and can be used as a very powerful tool that produces valuable results. However, success and swiftness of DNA typing of biological evidence either that found at a crime scene or used in disputed paternity testing, depends on the optimization of numerous factors. One of the most important and critical phases that ensures reliability of the whole procedure is the choice of the most suitable volume for the amplification protocol. Buccal swabs were collected from volunteers. DNA was extracted by Qiagen Dnaeasy™ Tissue Kit (Qiagen Co. 2001). PowerPlex 16 kit (Promega Corp., Madison, WI) was used to simultaneously amplify 15 STR loci by PCR. Amplification was carried out as described previously (Promega Corp. 2001). The tested total working reaction volumes were 5, 10 and 25µl. The PCR amplification was carried out in PE Gene Amp PCR System Thermal Cycler (ABI, Foster City, CA). Amplification products were analyzed on an ABI PRISM 377 instrument (ABI, Foster City, CA) in 5% bis-acrilamide gel. Amplification was generally successful for all the tested reaction volumes. Lower partial to complete DNA profiles ratio, the quality of obtained STR profiles, significantly reduced amount of reaction's components give advantage to 5µl reaction volume over other two tested volumes in this case.

KEY WORDS: forensic genetics, polymerase chain reaction, working volume, DNA profiling

## Introduction

Forensic genetics greatly benefited from the discovery of technique known as the polymerase chain reaction (PCR). This neat chemical reaction, first described in 1985 by Kary Mullis, has revolutionized molecular biology (1). PCR is an enzymatic process in which specific regions of DNA are duplicated repeatedly to yield millions of copies of a particular sequence in a matter of few hours (2,3). The procedure involves heating and cooling of chemical mixtures in a precise thermal cycling pattern. Hypothetically, approximately billion copies of the target DNA region are generated after 30 cycles. PCR reaction is a mixture of several components dissolved in DNA-free deionized water to reach preferred concentration of each constituent. The final component is the DNA template. The PCR process consists of three steps that are cyclically repeated. First, DNA strands are separated by heating (denaturation). Second, primers (DNA oligonucleotides) hybridize with target sites of these single stranded molecules to define the sequences to be amplified. Each primer performs as a starting point for the third step - the duplication of the target sequence. This last step occurs in the presence of the enzyme DNA polymerase and four nucleotide building blocks (4). STR loci, commonly used today in human DNA typing are usually assembled within one of commercial STR multiplexes. The availability of these STR kits that permit amplification of more than 10 STR loci has truly revolutionized forensic DNA analysis. Incredibly low matching probability (less than one in a billion) and possibility of successful amplification with only 1ng of DNA template promote these kits as powerful tools for various DNA testing applications (2). Also, the kits have significantly simplified preparation of PCR reaction. According to the manufacturers' recommendations, DNA templates just need to be added to a pre-made PCR mix containing all the necessary components for the amplification reactions. These kits are usually highly optimized by manufacturers. Any additional optimization is usually carried out by different user laboratories, according to their needs and purposes. An extremely important feature in the advanced DNA paternity testing, besides extremely high probability of paternity (>99,999%), is swiftness and simplicity of the whole method. Nowadays, as a result of intense optimization of the DNA isolation, amplification and detection procedures, the entire process can be performed within 6-7 hours. Hence, this type of testing is promoted to almost a standard practice in modern society. Our preliminary review represents small, but for our laboratory very valuable, contribution to optimization of forensic genetics procedures used in disputed paternity testing.

## Material and Methods

### SAMPLES COLLECTION

Six volunteers duly informed about the research donated DNA samples. Their buccal swabs were air-dried, sealed in 1,5 ml tubes, and immediately transported to the Laboratory for Forensic Genetics at the Institute for Genetic Engineering and Biotechnology, Sarajevo, B&H. The samples were stored at –20oC until DNA was extracted.

### DNA ANALYSIS

Qiagen Dnaeasy™ Tissue Kit was used for DNA extraction according to the manufacturer's protocol (5). PowerPlex®16 System (Promega Corp., Madison, WI) was used to simultaneously PCR amplify 15 autosomal STR loci and amelogenine. The STR loci are: D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX and FGA. Amplification was carried out as described previously (6) in total reaction volumes of 5, 10 and 25 μl. The PCR amplification was carried out in PE Gene Amp PCR System Thermal Cycler (ABI, Foster City, CA) according to the manufacturer's recommendations: 95°C (11 minutes), 96°C (1 minute), 10 cycles of: ramp 100% to 94°C for 30 seconds, ramp 29% to 60°C for 30 seconds and ramp 23% to 70°C for 45 seconds, 22 cycles of: ramp 100% to 90°C for 30 seconds, ramp 29% to 60°C for 30 seconds and ramp 23% to 70°C for 45 seconds, 60°C (30 minutes) and 4°C maintain. Amplification products were separated on an ABI PRISM 377 instrument (ABI, Foster City, CA) in 5% bis-acrilamide gel (Long Ranger® Single® Packs). Raw data were compiled and analyzed using the accessory software: ABI PRISM® Data Collection Software and Gene Scan®. Numerical allele designations of the profiles were obtained by processing with Powertyper™16 Macro. The threshold for the sample analysis was set at 100 rfu (relative fluorescence units).

### STATISTICAL ANALYSIS

Alleles were designated by comparison of the sample fragments with those of the allelic ladders provided with the kit. Statistical analysis included analysis of variance (ANOVA), followed by pair-wise comparisons with Newman-Keuls multiple comparison test, point bi-serial correlation coefficients, and simple linear regression conducted with *Winks 4.5 Professional* software (TexaSoft, Cedar Hill, TX, USA).

## Results

The characteristics of all the obtained STR profiles are presented in Table 1. The number of detected loci varies from eight to sixteen per generated PP16 STR profile. The weakest PP16 STR profile was detected for Sample 2 at 10 µl reaction volume. Only eight STR loci were detected for this reaction volume. The highest variation was observed within samples amplified in 10 µl reaction volumes. On the contrary, the highest fidelity was achieved with samples processed in 5 µl total volume – all the generated profiles were complete (16 loci). Means and variability measures (based on peak height) for the three tested volumes are showed in Table 2 and Table 3. Table 2 represents the measures obtained for each individual sample while Table 3 represents the same parameters based on joint results for each tested reaction volume. The calculated statistical parameters based on individual sample results as well as joint results clearly indicate that, at the 0,05 significance level, the means of 5 µl group is significantly different then other two (25 µl and 10 µl) groups.

| SAMPLES | TOTAL REACTION VOLUME (µl) | | |
|---|---|---|---|
| | 5µl | 10µl | 25µl |
| Sample 1 | 16/16 | 10/16 | 10/16 |
| Sample 2 | 16/16 | 8/16 | 13/16 |
| Sample 3 | 16/16 | 12/16 | 16/16 |
| Sample 4 | 16/16 | 14/16 | 16/16 |
| Sample 5 | 16/16 | 16/16 | 16/16 |
| Sample 6 | 16/16 | 16/16 | 15/16 |

TABLE 1. Results of nuclear STR profiling based of number of obtained loci in various reaction volumes

## Discussion

Application of STR loci in the interpretation of genetic profiles from different biological sources may be extremely valuable not only in a number of investigative procedures (routine paternity testing, forensics and mass disaster human identification), but also in different population studies (7). Numerous previous studies (8, 9, 10) describe PowerPlex®16 System (Promega Corp., Madison, WI) as a very powerful tool that produces valuable results. Previous Bosnian experiences confirm this (11-17). Therefore, we chose this STR multiplex system as a principal method for DNA typing performed in our laboratory. PCR is commonly performed in reaction volume range of 5-100 µl (2). Evaporation and accurate pipetting may cause a problem, or at least a challenge, with low volumes reaction. Conversely, larger reaction volumes encounter a problem of even heating and cooling throughout the entire volume. Therefore, in most molecular biology protocols PCR reaction volume is maintained in 20-50 µl range (2). According to the results of previous studies, manufacturer of PowerPlex®16 System (Promega Corporation) suggested total reaction volume of 25 µl, which was accepted and applied in earlier studies (8,9). Thus, in our earlier work we followed the same pattern (12). However, of recently, we have successfully used 10µl as the total reaction volume (15-17). Since the type of biological sample used in these studies was the same type that we usually employ in routine disputed paternity testing, we have decided to compare three different PCR total reaction volumes and select the one that suits our procedures best. Analyzing the number of the obtained STR loci for each sample we observed that the samples processed in 5 µl reaction volume resulted in complete profile. The fraction of detected loci per profile differs in other two tested volumes (10 and 25 µl). It is important to emphasize that RFU threshold was set at 100 units, and all peaks below the threshold, regardless whether they were detectable/useful or not were discarded (which was the case with most of the loci described as undetectable). Furthermore, calculated variability measures for different total reaction volumes of each sample, as well as the same parameters calculated for joint results clearly indicate that 5 µl volume significantly differs from 25 µl and 10 µl. The most illustrative is variability coefficient, which is significantly lower within 5 µl then 10 and 25 µl group. That means that the peak heights between different loci are better balanced and that the profiles obtained in 5 µl reaction volume are more transparent than those prepared in the other two volumes. Results obtained in this preliminary study suggest that, due to better ratio between the obtained complete and partial DNA profiles, quality of the obtained STR profiles and significantly reduced amount of reaction components, 5 µl total reaction volume is more suitable for using in these particular settings. Those would be our guidelines in our further examination that should include larger number of the examined samples and more relevant parameters (i.e. occurrence of stutter-peaks, balance of peak area etc.).

| | STATISTICAL PARAMETERS | LABEL | TOTAL REACTION VOLUME (µl) | | |
|---|---|---|---|---|---|
| | | | 5 | 10 | 25 |
| SAMPLE 1 | Mean | Blue (FL) | 2030 | 546,6 | 660,8 |
| | | Green (JOE) | 1418 | 115,7273 | 176,3636 |
| | | Yellow (TMR) | 1337,5 | 567,25 | 538,5 |
| | | All labels | 1606,828 | 388,8621 | 443,3103 |
| | Standard deviation | Blue (FL) | 1177,538 | 1038,61 | 1243,036 |
| | | Green (JOE) | 1201,002 | 164,9109 | 252,6279 |
| | | Yellow (TMR) | 455,1486 | 825,8732 | 728,7335 |
| | | All labels | 1054,189 | 757,7889 | 836,4723 |
| | Standard error of the mean | Blue (FL) | 372,3676 | 328,435 | 393,0797 |
| | | Green (JOE) | 362,1184 | 49,7229 | 76,1707 |
| | | Yellow (TMR) | 160,9209 | 291,9931 | 257,6487 |
| | | All labels | 195,7567 | 140,7169 | 155,328 |
| | Variability coefficient | Blue (FL) | 58,0068 | 190,0128 | 188,1108 |
| | | Green (JOE) | 84,6969 | 142,4996 | 143,2427 |
| | | Yellow (TMR) | 34,0298 | 145,5925 | 135,3266 |
| | | All labels | 65,6068 | 194,8734 | 188,6878 |
| SAMPLE 2 | Mean | Blue (FL) | 3189,5 | 408,1 | 583,7 |
| | | Green (JOE) | 2169,091 | 128,9091 | 153,4545 |
| | | Yellow (TMR) | 2489,286 | 569,4286 | 595 |
| | | All labels | 2613,571 | 338,6786 | 417,5 |
| | Standard deviation | Blue (FL) | 1756,509 | 862,6147 | 1177,029 |
| | | Green (JOE) | 1624,366 | 181,8232 | 214,521 |
| | | Yellow (TMR) | 1284,257 | 799,0041 | 846,6522 |
| | | All labels | 1606,096 | 660,0531 | 827,6109 |
| | Standard error of the mean | Blue (FL) | 555,453 | 272,7808 | 372,2066 |
| | | Green (JOE) | 489,7684 | 54,8222 | 64,681 |
| | | Yellow (TMR) | 485,3946 | 301,9896 | 319,9986 |
| | | All labels | 303,5238 | 124,7384 | 156,4038 |
| | Variability coefficient | Blue (FL) | 55,0716 | 211,3734 | 201,6496 |
| | | Green (JOE) | 74,8869 | 141,0476 | 139,7945 |
| | | Yellow (TMR) | 51,5914 | 140,3168 | 142,2945 |
| | | All labels | 61,4522 | 194,8907 | 198,2302 |
| SAMPLE 2 | Mean | Blue (FL) | 4139 | 554,2222 | 1235,889 |
| | | Green (JOE) | 2382,909 | 126,3636 | 392,3636 |
| | | Yellow (TMR) | 2873,75 | 890,125 | 1476,625 |
| | | All labels | 3087,607 | 482,1071 | 973,2857 |
| | Standard deviation | Blue (FL) | 1517,726 | 845,7867 | 1830,723 |
| | | Green (JOE) | 1557,167 | 196,7233 | 495,4627 |
| | | Yellow (TMR) | 1223,257 | 524,5086 | 833,6167 |
| | | All labels | 1597,77 | 632,6408 | 1224,612 |
| | Standard error of the mean | Blue (FL) | 505,9087 | 281,9289 | 610,241 |
| | | Green (JOE) | 588,543 | 59,3148 | 149,3887 |
| | | Yellow (TMR) | 432,4908 | 185,4436 | 294,7308 |
| | | All labels | 301,9502 | 119,5579 | 231,43 |
| | Variability coefficient | Blue (FL) | 36,6689 | 152,6079 | 148,1301 |
| | | Green (JOE) | 65,3473 | 155,6803 | 126,2764 |
| | | Yellow (TMR) | 42,5666 | 58,9253 | 56,4542 |
| | | All labels | 51,7478 | 131,2241 | 125,8225 |
| SAMPLE 4 | Mean | Blue (FL) | 4573,5 | 964,7 | 1377,9 |
| | | Green (JOE) | 3423 | 275,9 | 472 |
| | | Yellow (TMR) | 3654,571 | 2175,571 | 2115,857 |
| | | All labels | 3909,148 | 1023,519 | 1233,704 |
| | Standard deviation | Blue (FL) | 679,9675 | 1526,644 | 2142,26 |
| | | Green (JOE) | 1902,214 | 473,206 | 789,0451 |
| | | Yellow (TMR) | 1626,129 | 1555,542 | 1824,996 |
| | | All labels | 1516,872 | 1419,966 | 1735,907 |
| | Standard error of the mean | Blue (FL) | 215,0231 | 482,7638 | 677,4373 |
| | | Green (JOE) | 601,5286 | 149,6398 | 249,5162 |
| | | Yellow (TMR) | 614,6077 | 587,9288 | 689,771 |
| | | All labels | 291,9195 | 273,27 | 334,0724 |
| | Variability coefficient | Blue (FL) | 14,8676 | 158,2506 | 155,4728 |
| | | Green (JOE) | 55,5715 | 171,5136 | 167,1706 |
| | | Yellow (TMR) | 44,4958 | 71,5004 | 86,2533 |
| | | All labels | 38,8031 | 138,7337 | 140,7069 |
| | Mean | Blue (FL) | 4973,444 | 1644,111 | 1743,111 |
| | | Green (JOE) | 3544,273 | 553,0909 | 685,5455 |
| | | Yellow (TMR) | 3046,778 | 2413,333 | 1821,889 |
| | | All labels | 3833,414 | 1469 | 1366,414 |

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