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## Forensic Science International: Genetics

journal homepage: [www.elsevier.com/locate/fsig](http://www.elsevier.com/locate/fsig)Forensic massively parallel sequencing data analysis tool: Implementation of MyFLq as a standalone web- and Illumina BaseSpace<sup>®</sup>-applicationChristophe Van Neste<sup>a</sup>, Yannick Gansemans<sup>a</sup>, Dieter De Coninck<sup>a</sup>, David Van Hoofstat<sup>a</sup>, Wim Van Criekinge<sup>b</sup>, Dieter Deforce<sup>a,1</sup>, Filip Van Nieuwerburgh<sup>a,1,\*</sup><sup>a</sup> Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium<sup>b</sup> Biobix, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

## ARTICLE INFO

## Article history:

Received 16 August 2014

Received in revised form 19 September 2014

Accepted 3 October 2014

## Keywords:

Illumina

MiSeq

STR

Forensic loci

MPS

NGS

## ABSTRACT

Routine use of massively parallel sequencing (MPS) for forensic genomics is on the horizon. The last few years, several algorithms and workflows have been developed to analyze forensic MPS data. However, none have yet been tailored to the needs of the forensic analyst who does not possess an extensive bioinformatics background.

We developed our previously published forensic MPS data analysis framework MyFLq (My-Forensic-Loci-queries) into an open-source, user-friendly, web-based application. It can be installed as a standalone web application, or run directly from the Illumina BaseSpace environment. In the former, laboratories can keep their data on-site, while in the latter, data from forensic samples that are sequenced on an Illumina sequencer can be uploaded to Basespace during acquisition, and can subsequently be analyzed using the published MyFLq BaseSpace application. Additional features were implemented such as an interactive graphical report of the results, an interactive threshold selection bar, and an allele length-based analysis in addition to the sequenced-based analysis.

Practical use of the application is demonstrated through the analysis of four 16-plex short tandem repeat (STR) samples, showing the complementarity between the sequence- and length-based analysis of the same MPS data.

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## 1. Introduction

Obtaining forensic DNA profiles of polymorphic short tandem repeat (STR) loci using PCR followed by capillary electrophoresis (CE) is still the gold standard. However, routine use of massively parallel sequencing (MPS) for forensic genomics is on the horizon. MPS technologies do not rely on size separation and thus relieve the limitation on locus multiplexing that is present in CE [1,2]. MPS therefore creates enhanced possibilities within forensic genomics for analyzing degraded samples, mixed samples, and in dealing with kinship or population substructure [3,4].

Forensic bioinformaticians have been working on several algorithms to process MPS forensic STR data: lobSTR [5], RepeatSeq [6], STRait Razor [7], TSSV [8] and the MyFLq-framework [9]. LobSTR

and RepeatSeq are both genome wide STR aligners, and therefore outside of the scope of forensic analysis in its current legal and technological setting, in which targeted sequencing of a limited number of validated loci are investigated.

STRait Razor, TSSV and MyFLq are instead locus-centric, and operate on forensic loci. They require configuration information for each locus in the set, generally consisting of the repeat length of the locus, primer and/or flank sequences, and known alleles for the locus. All three programs have a similar approach to processing the STR data, which is represented in a flowchart in Fig. 1. To date, algorithms in these programs process data to the point of presenting allele candidates (step preceding the dashed red arrow in Fig. 1). It is at this point in the pipeline that data interpretation begins for the forensic analyst.

All current applications, are command-line based and are thus not well suited to be used by forensic analysts that do not have extensive bioinformatics experience. In this report, we present the MyFLq application that we developed into an open-source, web-based application with a user-friendly graphical user interface.

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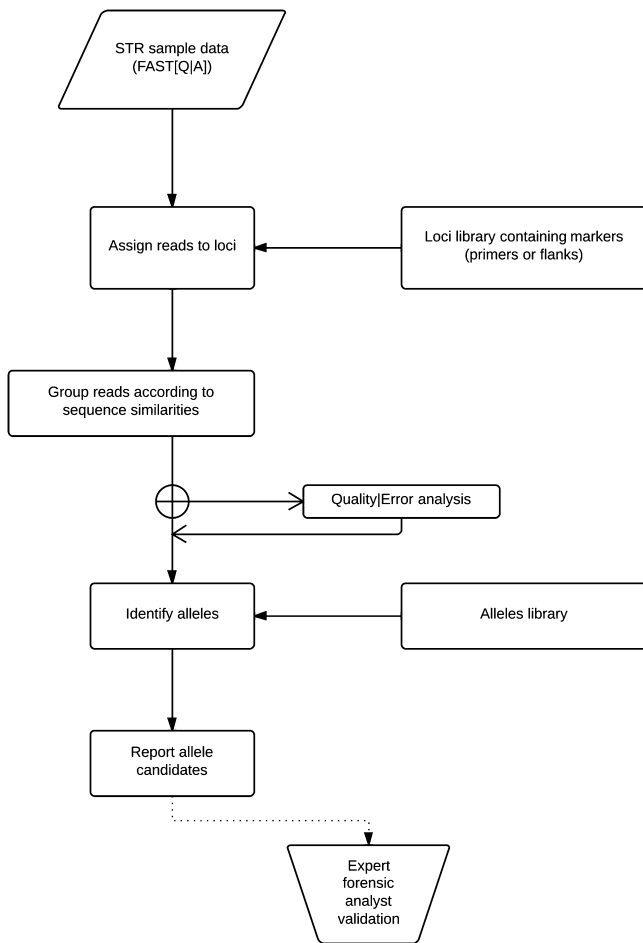


Fig. 1. STR data processing.

Additional features were implemented such as an interactive graphical report of the results, an interactive threshold selection bar, and an allele length-based analysis in addition to the sequenced-based analysis.

## 2. Materials and methods

MyFLq has been implemented both as a Django web application [10] and an Illumina BaseSpace application. Both implementations run from the same source code and users have access to the latest stable version, no matter the execution preference of the application. The BaseSpace MyFLq application requires no installation from the user. For the Django application, detailed documentation can be found on the MyFLq GitHub repository (<https://github.com/beukueb/myflq>). A pdf manual can be downloaded from <https://gitprint.com/beukueb/myflq>, covering both implementations. The development version and previous builds are only available for the Django application.

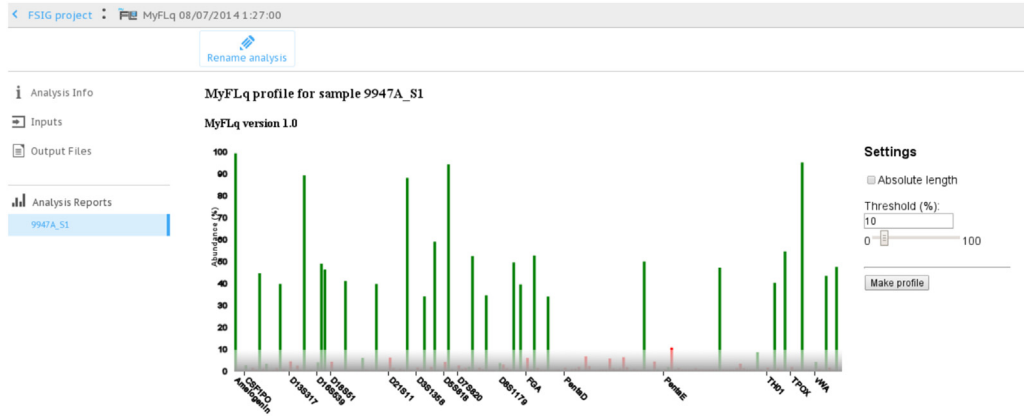
### 2.1. Samples

The same data were used as in the MyFLq framework paper [9]. The results presented in this report were obtained with sample 9947A\_S1, which is a single contributor control DNA sample (Promega) [11]. This sample was amplified using a 16-plex PCR, based on the PowerPlex<sup>®</sup> 16 primers (Promega) [12]. The reference profile for 9947A with the 16-plex is shown in Supplementary Table A.1. The MyFLq framework paper [9] also analyzed a second single contributor sample and two multiple person mixtures. Results for these samples are available on BaseSpace, together with the FASTQ data for anyone wishing to experiment with MyFLq.

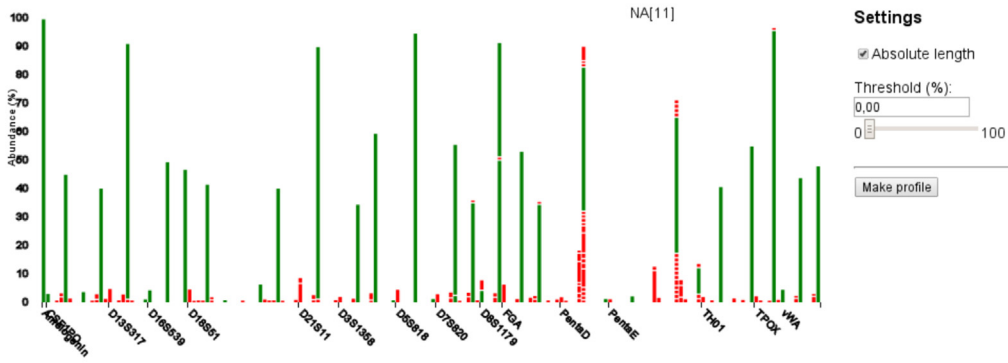
### 2.2. Launching MyFLq

To produce the results for this report, MyFLq was launched from <http://basespace.illumina.com/apps>. A threshold of 0.5% was set to filter read groups with a lower abundance for further analysis. The loci set and the allele database were set to the MyFLq framework paper options, as shown in Fig. 2. The database contained all the alleles from the framework paper's four DNA samples, including sample 9947A [9]. The database consists of all sequences of the Powerplex<sup>®</sup> 16 alleles present in these four samples. For the other options the default values were used. Detailed information on these settings can be found in Supplementary Table A.2 or the online documentation. A BaseSpace project "FSIG" was made to which the results could be saved. Finally, the analysis was launched by clicking "Continue".

Fig. 2. Primary analysis settings. The setting "Select loci set" controls the configuration of the set of loci that will be analyzed in the dataset. Normally these are the same loci as in the applied STR multiplex PCR. With the "Select allele database" setting, the database with the reference alleles sequences can be configured. Both configurations can be provided as csv-files if they are not available in the dropdown menus. Settings on the right are general and can be used to tweak the results. They are described in Supplementary Table A.2.



(a) Initial display of analysis result.



(b) Alleles proportionally sorted and stacked according to length.

Fig. 3. Initial display and proportionally sorted analysis result.

### 3. Results

#### 3.1. Initial sequence-based result display

Fig. 3a shows the analysis result page, that can be found under the project folder where the analysis was saved. The initial display shows an interactive visual representation that should be interpreted as a sequence-based analysis rather than a length-based analysis. The different bars represent grouped allele sequences and are sorted according to length. Spacing is however not proportional and allele candidates of the same length are not stacked on top of each other, but rather side-by-side. A green bar is

given to sequences that are present in the database, a red bar when not. The vertically adjustable gray transparent zone determines the threshold for which allele candidate bars with a lower abundance will not be withheld in the final profile. By default, it is set to 10%. Note that sequences with an abundance threshold lower than 0.5% (configurable) are already filtered during the analysis.

#### 3.2. Detailed allele candidate information

When hovering over a bar, a detailed block of information is displayed for that allele candidate. An example is shown in Fig. 4. This information can be used to examine if the underlying

#### Locus D8S1179 → allele candidate: 13[b]

Locus stats		Allele stats	
Total reads	84546	Index	5
Filtered reads	79424	Abundance	39.76%
Total unique	368	Strand distribution	49.17%
Filtered unique	7	Clean flanks	96.3%

In profile:

#### Relations to other sequences within D8S1179

Allele index	Relation degree	Sequence
5	-	ATC <b>A</b> CTATCTATCTATCT
1	I'st	ATC <b>A</b> CTATCTATCT
4	I'st	ATC <b>A</b> CTATCTATCTATC
6	I'st	ATC <b>A</b> TTTTATCTATCTATCT
7	I'st	ATC <b>A</b> CTATCTATCTATCT
2	II'nd	ATC <b>G</b> TCTATCTATCT
3	II'nd	ATC <b>G</b> TCTATCTATCTATC

#### Locus D8S1179 → allele candidate: 13[a]

Locus stats		Allele stats	
Total reads	84546	Index	7
Filtered reads	79424	Abundance	49.78%
Total unique	368	Strand distribution	48.75%
Filtered unique	7	Clean flanks	96.2%

In profile:

#### Relations to other sequences within D8S1179

Allele index	Relation degree	Sequence
7	-	ATC <b>G</b> CTATCTATCTATCT
1	I'st	ATC <b>A</b> CTATCTATCTATCT
2	I'st	ATC <b>G</b> TCTATCTATCT
3	I'st	ATC <b>G</b> TCTATCTATCTATC
5	I'st	ATC <b>A</b> CTATCTATCTATCT
4	II'nd	ATC <b>A</b> CTATCTATCTATCTATC
6	II'nd	ATC <b>A</b> TTTTATCTATCTATCT

Fig. 4. Information blocks for true D8S1179 alleles. Their one-base sequence difference is indicated by dotted lines.



automatically filtered by the 10% default threshold. The information supports that this candidate allele should be disregarded. The putative allele length is one STR repeat unit smaller than the high abundant (47.40%) sequence with index 6, indicating that it might be stutter. Apart from this stutter there are no other sequence differences (1st relation degree). Furthermore, the clean flank percentage is rather low (59.5%), indicating possible low quality sequences. An unexpected strand distribution of 100% implies that there are no complementary reads supporting the presence of this allele candidate. Removing this allele candidate is accomplished by unchecking the “in profile” check-box.

### 3.3. Allele candidates proportionally sorted according to length

After selecting the “Length-based analysis” check-box, all allele candidates are displayed proportionally, according to their actual length within the locus, as shown in Fig. 3. For each locus, the x-axis is adjusted to show the locus length starting from the shortest allele and ending at the longest allele. The threshold bar is no longer displayed because allele candidates with the same length are now stacked on top of each other, which creates one bar that shows the total abundance of all alleles with the same length within each locus. This representation resembles a CE profile. The example of the allele candidate in Fig. 5 now visually looks like a CE stutter peak based on the relative length and abundance difference as compared to the true allele.

### 3.4. Final profile

After reviewing the profile by setting the threshold to an appropriate value, and removing allele candidates of poor quality,

Locus	Alleles
Amelogenin	X
CSF1PO	10[a],12[a]
D13S317	11[b]
D16S539	11[a],12[a]
D18S51	15[a],19[a]
D21S11	30[a]
D3S1358	14[a],15[b]
D5S818	11[a]
D7S820	10[b],11[a]
D8S1179	13[a],13[b]
FGA	23[a],24[a]
PentaD	12[a]
PentaE	12[a]
TH01	8[a],9.3[a]
TPOX	8[a]
vWA	17[a],18[a]

Fig. 6. Final profile for sample 9947A\_S1 with 10% threshold.

pressing the “Make profile” button yields the final profile. This profile can then be used to query databases or compare to the profile of a sample of interest. Fig. 6 shows the final profile for sample 9947A\_S1. Using the threshold of 10%, it has one Penta E allele 13 that is undetected relative to the known genotype (Table A.1). This allele is present in the data at an abundance of 8.85% and its corresponding green bar can be seen clearly in Fig. 3. The sub-optimal results of the pentanucleotide loci, Penta D and Penta E, were previously discussed in detail [9].

## 4. Discussion

We show how an MPS data-set can be analyzed using an easy-to-use graphical user interface, requiring a limited number of parameters and almost no bioinformatics expertise. The interactive visual representation of the results shows additional information when hovering over the alleles, allowing for in-depth analysis of the underlying sequences and the related statistics. For clarity of explanation we chose to display and discuss the analysis of a single contributor sample, but the MyFLq framework equally works on mixtures because no assumptions on mixture composition are made to perform the analysis. The main added value of MPS over CE indeed lay in the analysis of mixed and degraded samples [9]. With MPS, sequences can be analyzed more in depth to determine whether they are genuinely from one of the original contributors of a sample, or instead more likely to be the product of a PCR or sequencing error. Additionally, due to the ability to multiplex more loci than CE affords, broader genetic interrogation can be achieved in a single reaction, thus conserving precious samples.

The reported results comprise only 16 loci, but MyFLq can run with any number of loci. When running MyFLq with a custom loci set, the primers of these loci can be imported. The allele database is not strictly necessary to run the program. In exploratory studies, for example if building a database of known alleles, MyFLq can be run with an empty allele database. The GitHub repository contains example files for users that need either a custom locus set or custom allele database.

The used allele database was very small as it only compromised the alleles of the five contributors. Sequences that are currently not in the database are marked as red bars. These bars are very useful to visually monitor the noise level. In the future, with a larger database, it could be that erroneous sequences are nonetheless present in the database, as they could be true alleles for individuals that are not present in the sample. The solution to that problem could be to mark rare alleles (e.g. alleles with a population prevalence <1%) with a different color. The combination of unknown alleles and rare alleles would then indicate the level of noise. A further limitation of the current database is its nomenclature. Currently same-sized alleles get an arbitrary name within the database, which would make it difficult to perform searches in other databases without the original sequence. When an international nomenclature for MPS STR alleles has been established, it will be incorporated in MyFLq.

When all allele candidates have been reviewed, the “Make profile” action generates a report with only the selected alleles. This is the profile that a forensic analyst can use to either store in a database, to query against a database, or for direct comparison to a known sample of interest. Future versions of the software will include possibilities to interact directly with sample databases. New feature requests can be made through the GitHub website.

## 5. Conclusion

MyFLq is the first open-source, web-based forensic MPS DNA analysis software with an easy-to-use graphical user interface.

It can run natively on Illumina BaseSpace, or independently on a forensic laboratory's server. The possibility to run the program directly from the Illumina BaseSpace environment means no extensive bioinformatics skills are required.

### Competing interests

C.V.N. participated in an internship program at Illumina, Inc. to provide feedback on building a native BaseSpace application to the Illumina developers.

### Acknowledgments

Funding was provided by the Ghent University Multidisciplinary Research Partnership 'Bioinformatics: from nucleotides to networks'

The authors would also like to thank Illumina, Inc. for providing the data and making MyFLq easily accessible on their BaseSpace platform.

### 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.fsigen.2014.10.006>.

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