HITACHI
Genetic Systems
Hitachi Software Engineering Co., Ltd.

APPLICATION NOTE

Human Identification Using the FMBIO® II Fluorescence Imaging System

Introduction

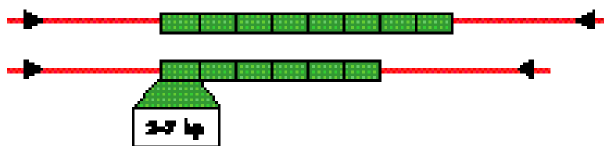
PCR-based DNA assays have provided the forensic community with several new tools to assist in individual human identification. With previous identification methods, it was impossible to genotype samples which were old, degraded, or limited in quantity. PCR-based typing methods have greatly reduced these limitations, significantly shortened the time required to complete an analysis, and eliminated the safety considerations of working with radioactivity. PCR-based analysis systems have become widely accepted in the fields of forensic and parentage testing due to their sensitivity and robustness.[1]

STR Analysis for Individual Identity and Databanking

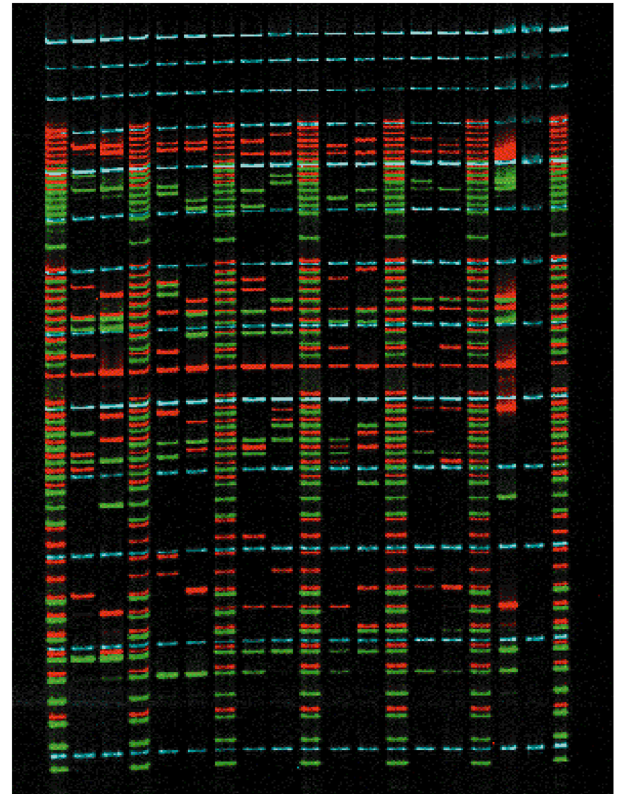
Short tandem repeats (STRs) are short, repetitive DNA sequences two to seven base pairs long,[2-7] with alleles differentiated by the number of times a sequence is repeated. STR loci may be detected by polymerase chain reaction amplification using labeled primers (Fig. 1).[8] Electrophoretic separation is then used to distinguish alleles by size (Fig. 2).

STRs are abundant and widely distributed throughout the human genome. They are well characterized and highly polymorphic, making them ideal for use in individual human identification.[9,10]

The discriminatory power of STR analysis is greatly enhanced by evaluating samples at more than one locus simultaneously (multiplexing). When comparing forensic samples at eight loci, as shown in Fig. 2, matching probabilities can exceed 1 in 118,000,000. In parentage investigations, multiple-locus analysis can result in paternity probabilities of 0.9979 and higher.[11]



1 **Schematic diagram of the STR amplification process.** Primers upstream and downstream of the repeat sequences are used to amplify product. Amplification products vary in size, depending on the number of repeated elements.

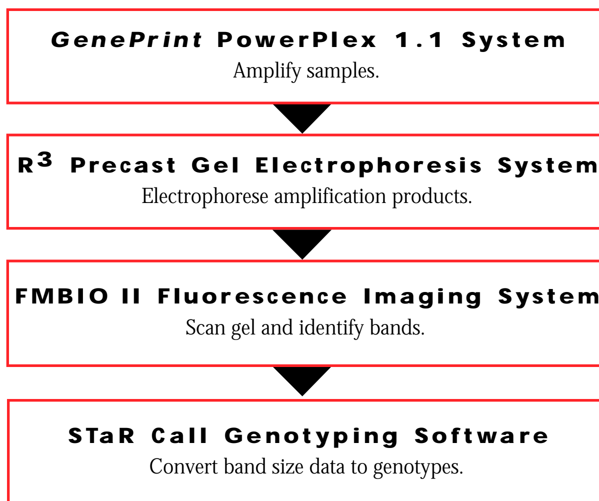


2 **Multicolor fluorescence imaging.** FMBIO Analysis Software builds multicolor images by displaying data collected through each band-pass filter in a separate color. Files are superimposed to display a multicolor image.

STR data are efficiently analyzed using a unique combination of instrumentation, non-isotopic chemistry, and software that makes processing DNA samples fast, safe, and easy for human identity applications and databasing (Fig. 3).

Amplification of STR Loci

The *GenePrint*™ PowerPlex™ 1.1 System (Promega Corp., Madison, WI) is a three-color fluorescent STR kit which effectively utilizes the multicolor capability of the FMBIO. The kit allows simultaneous amplification of eight human polymorphic STR loci in a single tube. For four of the loci, one primer is labeled with carboxy-tetramethylrhodamine (TMR); for the other four loci, one primer is labeled with fluorescein. Amplification of 1-25 ng of human template DNA results in fluorescently labeled products between 100 and 350 base pairs in length. By labeling products in different colors, bands of similar sizes can be easily distinguished from one another.

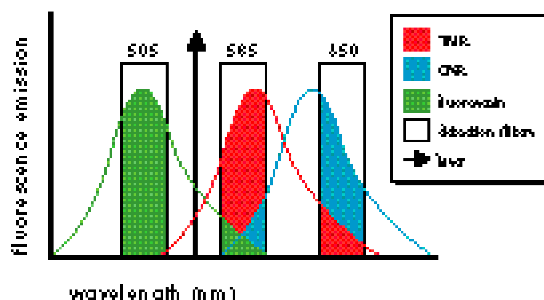


3 **Using the FMBIO II Fluorescence Imaging System in STR data processing**. The combination of multicolor STR kits, precast gels, a fluorescence imager and genotyping software simplifies the STR analysis process.

To mitigate the effects of lane-to-lane mobility variations, Promega has developed the Fluorescent Ladder (CXR), 60-400 Bases. This product is a size ladder labeled with carboxy-X-rhodamine (CXR). The ladder contains bands ranging from 60 to 400 bases in length, and can be mixed with amplification products before electrophoresis to create an in-lane size standard.

Electrophoresis of STR Products

After amplification, samples are electrophoresed on a polyacrylamide gel. Hitachi's R3™ Precast Gel Electrophoresis System provides fast, easy separation of PowerPlex products. R3 gels are designed to take advantage of the full scanning area of the FMBIO, providing maximum separation distance for samples. The 4.5% polyacrylamide 7M urea gels are precast and disposable, thus saving the analyst time and effort in cleaning plates, pouring gels, and waiting for acrylamide polymerization.



4 **Schematic diagram of three-color detection using the FMBIO.** Photons of light energy are supplied at 532nm. Energy is absorbed by the fluorescent dyes, which become excited, and then emit light at the wavelength ranges shown for fluorescein (green), carboxy-tetramethylrhodamine (red), and carboxy-X-rhodamine (blue). Rectangles indicate the approximate wavelengths of light isolated as separate images by the 505, 585, and 650 nm band-pass filters. Images acquired through each of these filters are overlaid to create a three-color image, as shown in Fig. 2. Light captured from adjacent dyes may be reduced using the proprietary color separation matrix in the FMBIO Analysis Software.

Instrumentation for Detecting STR Bands

Fluorescence is considered the state-of-the-art detection method for STR because it offers multicolor analysis capability for PCR amplification products. Fluorescence also enables direct detection of PCR products, provides excellent detection sensitivity, and is rapid, thus providing the high throughput desired for processing samples for forensic databases.

The FMBIO® II Fluorescence Imaging System is a laser-based, fluorescence scanner featuring a 20 mW solid-state yttrium aluminum garnet (YAG) laser which emits light at an excitation wavelength of 532 nm. The instrument also features two photomultiplier tubes, a large scan area of 20x43 cm, and a linear dynamic range of four orders of magnitude.[12] The FMBIO produces extremely high resolution images capable of resolving even single-base microvariants.

Multicolor imaging is achieved using band-pass detection filters to discriminate light from fluorescent dyes emitting between 500 and 700 nm (Fig. 4). Up to four filters can be stored in the instrument and accessed through software. Two filters can be used simultaneously to detect emissions from two different dyes.

In three-color analyses, the gel is scanned after electrophoresis using a 505 nm band-pass filter to detect fluoresceinated amplification products, a 585 nm band-pass filter to detect products labeled with TMR, and a 650 nm band-pass filter to detect the CXR-labeled internal size standards. These images can be overlaid into a three-color image (Fig. 2) or viewed separately (Fig. 5).

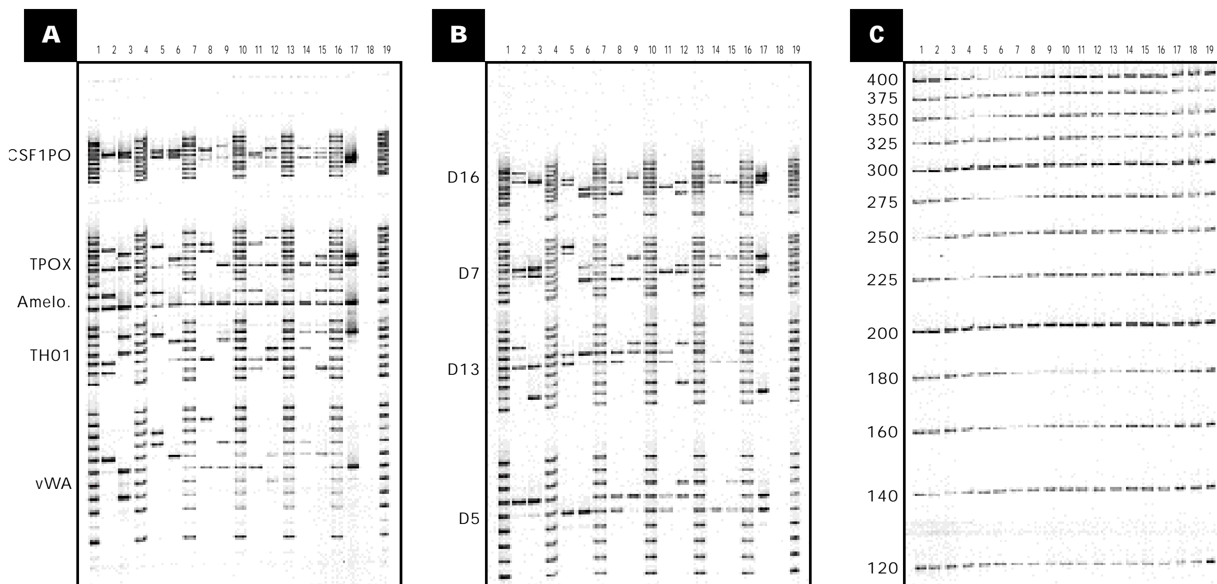
The FMBIO requires only 15-20 minutes to scan two dyes at once. Since gels are scanned after electrophoresis has been completed, one instrument can process many more gels per day than scanners that are dedicated to a single electrophoresis unit.

Software for Analyzing Genotypes

The FMBIO II Fluorescence Imaging System includes a computer and three powerful software programs.

ReadImage controls the FMBIO scanning unit as it generates digital images of gels. ReadImage is used to easily set the scan area, scan resolution, image orientation, and photomultiplier sensitivity. The user can also choose filters, add comments to be saved with the scanned image, and perform hardware checks. The experimental data collected are converted by ReadImage into 16-bit digital TIFF files for analysis with FMBIO Analysis Software. For multicolor images, ReadImage creates a folder in which all TIFF files generated are stored together.

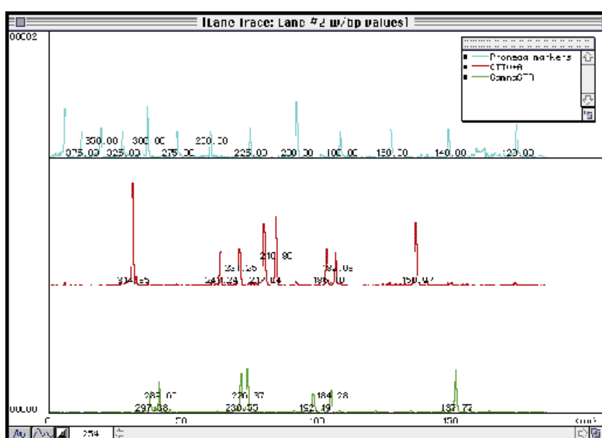
FMBIO® Analysis Software offers a user-friendly interface for viewing and analyzing images. The software features analysis functions such as automatic band calling to facilitate data processing, quantitation of peak height or peak area, and band sizing through logarithmic comparison to size standards. Data can be displayed as either gel



5 **FMBIO scan of the *GenePrint* PowerPlex 1.1 System.** Eight loci plus amelogenin (a gender marker) were co-amplified, mixed with size standard, separated by electrophoresis, and imaged on the FMBIO II Fluorescence Imaging System. Human DNA (2 ng) was amplified in each PCR reaction. Lanes 1, 4, 7, 10, 13, 16, and 19: allelic ladder. Lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, and 15: amplified human STR loci. Lane 18: negative control. All lanes contain CXR-labeled internal size standards. **Panel A:** One primer for each of the CTTv loci (CSF1PO, TPOX, TH01 and vWA) plus amelogenin was labeled with TMR; the image was acquired using a 585 nm band-pass filter. **Panel B:** One primer for each of the GammaSTR loci (D16S539, D7S820, D13S317, and D5S818) was labeled with fluorescein; the image was acquired using a 505 nm band-pass filter. **Panel C:** Size standards (400, 375, 350, 325, 300, 275, 250, 225, 200, 180, 160, 140, and 120 bp) were labeled with CXR; the image was acquired using a 650 nm band-pass filter. For a multicolor image, all three images are overlaid, as shown in Fig. 2.

images (Fig. 2 and 5) or electropherograms (Fig. 6). This option provides more flexible data analysis by allowing visual inspection of raw data, a feature not available with other fluorescent instruments and software packages.

STaR Call™ Genotyping Software performs automated genotype calling and analysis of artifact ("stutter") bands. Band sizes calculated by FMBIO Analysis Software are imported into STaR Call as Paste Values. STaR Call then compares these values with a selected STR Lookup Table which contains size ranges for each allele. Based on comparison of band sizes with allele ranges, bands are assigned a locus name and repeat number (Fig. 7). Weak bands occurring in the stutter position are identified as artifacts and are excluded from the final output. Genotype data generated by STaR Call are easily converted to common message format (CMF) files for export to the Combined DNA Index System (CODIS) forensic database.



6 **Sample FMBIO Analysis Software output.** Band position and peak intensity data from Lane 2 of the gel in Fig. 2 and 5 are displayed graphically. Information from each of the three channels is shown as a separate graph. Band sizes (bp) are calculated by FMBIO Analysis Software based on the known sizes of internal size standards (shown in blue). Peaks representing homozygous genotypes are roughly twice the height of heterozygous genotypes. Information can also be overlaid to generate a composite electropherogram.

Lane2

bp	Genotype		OD		Percentages
314.85	CSF1PO	12	12016		
311.01	CSF1PO	11	818		6.81%
243.24	TPOX	11	4138		
231.25	TPOX	8	4212		
217.04	Y		6793	Y	
210.80	X		7131	X	
186.10	TH01	7	3524		
182.08	TH01	6	3362		
150.97	vWA	17	6312		
146.99	vWA	16	378		5.99%

Lane2

bp	Genotype		OD	Percentages
297.38	D16S539	14	1575	
293.79	D16S539	13	112	7.11%
289.66	D16S539	12	1763	
230.55	D7S820	10	1950	
226.37	D7S820	9	2329	
192.19	D13S317	13	1193	
188.24	D13S317	12	12	1.01%
184.28	D13S317	11	1177	
137.77	D5S818	12	2178	
133.91	D5S818	11	26	1.19%

7 **Sample STaR Call Genotyping Software output.** Band sizes (bp) are converted into genotypes by the STaR Call program. In this example, the individual analyzed has a genotype of CSF1PO 12 (homozygote); TPOX 8,11; X,Y (male); TH01 6,7; vWA 17 (homozygote); D16S539 12,14; D7S820 9,10; D13S317 11,13; D5S818 12 (homozygote). Quantitative information (OD) is evaluated to determine the presence of artifact ("stutter") bands. Percentages represent the ratio of the stutter band to the major band above it. Stutter bands appear in the CSF1PO 11, vWA 16, D16S539 13, D13S317 12 and D5S818 11 positions.

Conclusions

Recent important advances can substantially improve STR data analysis in the following ways:

Fewer amplification steps. The *GenePrint* PowerPlex 1.1 System amplifies eight loci in one tube, reducing the number of reactions per DNA sample.

Less preparation time. Hitachi's R^3 Precast Gel Electrophoresis System eliminates the time required to clean and prepare glass plates, and to prepare, pour and polymerize acrylamide.

Fewer gels. Because amplification products are labeled in multiple colors, bands of similar sizes are easily distinguished, thus increasing the number of loci that can be separated on each gel.

More samples per gel. Internal size standards correct for lane-to-lane mobility variations, reducing the number of allelic ladders loaded per gel, and increasing the number of lanes available for samples.

More samples per day. The FMBIO II Fluorescence Imaging System captures an image after electrophoretic separation. Two different dyes are scanned in only 15-20 minutes. Many more samples can be processed per day compared to instruments that perform imaging during electrophoresis.

Less time processing data. Analysis is extremely flexible because data can be displayed as a gel image or as band position and intensity in graphical form. The automatic allele calling and databanking capabilities of STaR Call Analysis Software eliminate the need for data entry, saving time, and eliminating the possibility of data entry errors.

Acknowledgments

We thank Jim Schumm and Ann Lins of Promega Corp. for their suggestions, images, and diagrams.

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