

Multilocus Variable Number Tandem Repeat

Genotyping of

Mycobacterium tuberculosis

Technical Guide

86	2	2	5	3	2	5	1	5	3	3	2	3	2	3	2	3	6	8	3	4	4	2	3
87	2	2	3	3	2	5	1	4	3	3	2	3	2	3	2	3	6	2	3	4	4	2	3
88	2	2	7	2	2	5	1	1	3	2	2	3	3	5	4	3	9	2	4	4	4	2	3
90	2	2	3	3	2	5	1	7	3	2	3	3	2	4	4	2	12	6	4	4	4	2	3
92	2	3	4	4	2	4	2	2	4	2	2	1	3	4	3	2	5	8	2	3	4	2	2
93	2	5	4	3	2	11	2	2	3	6	3	3	3	4	4	8	6	3	5	3	1	4	3
95	2	2	4	2	2	6	1	5	3	3	2	1	5	4	4	3	2	4	2	4	1	1	5
96	2	2	3	1	2	5	1	5	3	3	2	2	2	4	2	2	5	3	2	4	2	2	3
97	2	1	3	3	1	5	1	2	3	4	2	3	3	4	1	3	5	2	4	4	2	1	3
98	2	2	4	2	2	6	1	6	3	3	2	1	4	2	4	4	2	4	2	4	1	2	3

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To C., E., C., A. and A.

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Preface

What you should know before beginning:

This manual is written for principle investigators and laboratory staff who are planning to perform Variable Number Tandem Repeat genotyping of *Mycobacterium tuberculosis* complex isolates, using agarose gel electrophoresis or using a 3100 Genetic Analyzer. Although the general principles are the same, some adaptations may be needed for 3130, 3100 Avant, and 3700 and/or other software versions.

Before attempting the procedures in this manual, you should be familiar with the following topics:

- General techniques and safety procedures for obtaining and handling *Mycobacterium tuberculosis* complex isolates.
- General techniques and safety procedures for handling DNA samples, performing PCR, preparing PCR products for electrophoresis, and performing electrophoresis.
- 3. Windows operating systems
- The basics for installing, operating and maintaining a 3100 Genetic Analyzer. Detailed information for these procedures are given in Applied Biosystems' manuals.

1 Introduction

Variable Number Tandem Repeat (VNTR) sequences have emerged as valuable markers for genotyping of several bacterial species, especially for genetically homogeneous pathogens such as *Bacillus anthracis* (1, 2), *Yersinia pestis* (2, 3) and the *M. tuberculosis* complex members (see below). VNTR genotyping basically rely on PCR amplification using primers specific for the flanking regions of the VNTRs and on the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, these sizes reflect the numbers of the amplified VNTR copies. The final result is a numerical code, corresponding to the repeat number in each VNTR locus. Such numerical genotypes are intrinsically portable and are thus particularly convenient for both intra- and inter-laboratory comparative studies (4, 5). In addition, compared to IS*6110*-RFLP, MIRU-VNTR typing has the advantages of being faster, and appropriate for virtually all *M. tuberculosis* isolates, including strains that have a few IS*6110* copies (4, 5).

Initial VNTR typing systems for *M. tuberculosis* complex strains made use of very limited sets of loci (4, 6-9), which turned out to not be sufficiently discriminatory (10). More extensive sets of VNTR loci have been described subsequently (11-15), including a system based on 12 loci (14), which has been shown to be applicable for reliable genotyping and molecular epidemiology studies of *M. tuberculosis* (5, 16). These loci contain VNTR of genetic elements named Mycobacterial Interspersed Repetitive Units (MIRUs) that are located mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome (14, 16, 17). As the other VNTRs sequences mentioned above, the lengths of MIRU repeat units are in the range of 50-100 bp, and belong therefore to the "minisatellite" VNTR category (16). <u>All above loci are collectively designated as MIRU-VNTR</u> loci in this guide.

A MIRU-VNTR-based high-speed genotyping system has been developed, which combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping (16). Both this system and the simpler system using electrophoresis with agarose gels are highly reproducible at intra- and inter-laboratory levels (14, 18). A recent population-based study indicated that the use of the 12 locus-based MIRU-VNTR typing as a first-line method, together with spoligotyping, provides adequate discrimination in most cases for large-scale, prospective genotyping of *Mycobacterium tuberculosis* in the United States. IS*6110* fingerprinting can subsequently be used as a secondary typing method to type the clustered isolates, when additional discrimination is needed (19). Other studies have shown the interest of this typing method for clinical mycobacteriology (*e.g.* 20) or for local outbreak investigation (21).

In order to further reduce the numbers of isolates to be analyzed by IS6110 fingerprinting, we have recently evaluated the additional information provided by a set enlarged to 29 loci, using a total of 824 tubercle bacillus isolates, including representatives of the main lineages identified worldwide so far (Supply et al., in preparation). Five loci (i.e. QUB-3232, -3336, -1895, -18 (alias VNTR 1982), -11a (alias VNTR 2163a)) were excluded for lack of robustness and/or stability in serial isolates or isolates from epidemiologically-linked patients. The use of the 24 remaining loci increased the number of types by 40% - and by 23% in combination with spoligotyping - among isolates from cosmopolitan origins, as compared to those obtained with the original set of 12 loci. Consequently, the clustering rate was decreased by four-fold – by three-fold in combination with spoligotyping - under the same conditions. A discriminatory subset of 15 loci with the highest evolutionary rates was then defined, that concentrated 96 % of the total resolution obtained with the full 24-loci set. Its predictive value for evaluating M. tuberculosis transmission was found to be equal to that of IS6110 RFLP typing, as shown in a companion population-based study. This 15-loci system is therefore proposed as the new standard for routine epidemiological discrimination of M. tuberculosis isolates, and the 24-loci system as a high-resolution tool for phylogenetical studies (Supply et al., J. Clin. Microbiol., in press).

A table giving the correspondence between different nomenclatures for the 24 loci retained is given in Appendix 4.1.

2 Methods

Note: Aerosol resistant pipet tips are used at all experimental steps before PCR amplification, to avoid potential contamination problems.

2.1 DNA extraction and dilution

2.1.1 Principle

As it is based on amplification by PCR, MIRU-VNTR typing can be performed on heat inactivated mycobacterial colonies or mycobacterial pellets from liquid cultures without extensive DNA purification. It can be applied to various biological materials, including non-viable material, permitting for instance retrospective analyses of stocks of non-viable cells.

Note: alternatively, purified DNA obtained by the internationally standardized method as described by van Soolingen *et al.* (22) or by other standard methods for IS*6110*-RFLP analysis can also be used.

2.1.2 Procedure

- Resuspend mycobacterial colonies grown on solid media (*e.g.* Löwenstein-Jensen) medium or mycobacterial pellets obtained from liquid cultures (*e.g.* MGIT) into 200 μl 10 mM Tris-HCl, 1 mM EDTA (pH 7.0), in a screwed cap tube or a safe lock tube.
- For colonies recovered from solid media, go to step 3. Re-centrifuge mycobacterial pellets obtained from liquid cultures at 15,000 g x 5 min, discard the supernatant and resuspend the bacterial pellet into 200 μl 10 mM Tris-HCl, 1 mM EDTA (pH 7.0).
- Incubate at 95°C for 45 min, using an oven or a PCR cycler with a hot lid, if available in the microbiological security facility.
- 4. Centrifuge the suspension at 15,000 g x 1 min, to pellet the cell debris.
- 5. Harvest the supernatant containing the DNA and transfer into a new tube.
- Store concentrated stocks at -20 °C until further use, or dilute at 1:50 into sterile water in a new tube. Diluted solutions can be also stored at -20 °C.

2.2 PCR amplification

2.2.1 Principle

PCR amplification of different VNTR regions is performed using primers specific for the flanking regions of each VNTR region.

When analysis of the PCR products is done using a DNA sequencer, 8 different multiplex PCRs are performed in order to analyze eight groups of three loci simultaneously. For each multiplex, one primer per oligonucleotide pair is tagged with a specific fluorescent dye (appendix). In case of amplification failure of some loci in some multiplex reactions, these loci are then usually amplified separately by simplex PCR, in a second round.

When analysis of the PCR products is done using only electrophoresis with agarose gels, separate amplification of each locus is performed, using unlabeled oligonucleotides.

2.2.2 Procedure

2.2.2.1 Preparation of a PCR spreadsheet

- Prepare a PCR spreadsheet, indicating the position of each sample in the PCR 96well microplate (Figure 1).
- 2. If different multiplex or simplex PCRs are performed in a same microplate, use specific extensions after sample names (e.g. sample 1-mix1) or use different colors to specify the different reactions, as shown in the example below. Samples analyzed with the same multiplex or the same locus should be grouped together for convenience.
- 3. Include positions for a negative control (sterile water) and a positive control (H37Rv or BCG Pasteur) for each multiplex or simplex set, to validate the analysis.

Figure 1 PCR spreadsheet model

Н	G	F	Е	D	С	В	А	
							Sample 1	1
							Sample 2	2 3
								3
								4
								4 5 6
								6
								7
								8
								9
								10
								11
								12

Colour code:



2.2.2.2 Preparation of PCR premixes

PCR premixes can be prepared extemporaneously just before the addition of genomic DNA, or stock solutions can be prepared, aliquoted and stored at -20°C until further use. The final concentration of MgCl₂ varies from 1.5 mM (default concentration using the 10 X buffer) to 3 mM (by including additional MgCl₂), according to the multiplex or the MIRU locus. The final volume per reaction (after addition of DNA) is 20 µl.

The use of Oiagen Hotstart Taq Polymerase kit including O solution is strongly recommended. If this kit is not used, PCR failures may be observed, especially for MIRU-VNTR locus 20, and more intense stutter peak ladders (see below) may be seen, leading to possible misinterpretation problems. Note: the Qiagen PCR Multiplex PCR kit may be specifically used for mix 5, to reduce pronounced stutter peaks seen with large alleles of locus 4052 (see Annex 5).

Using our conditions, Q solution is the limiting reactive in the Qiagen kit. However, the use of Betaine (identical to Q coumpond, according to Sigma) at a final concentration of 1M in replacement of Q solution is a good and cheap alternative.

 In a DNA-free area, prepare the PCR reaction premixes for the different multiplex and simplex reactions, according to Table 1, Table 2, and Table 3. Important: to take into account void volumes, prepare a 5-10 % proportion in excess of the volume needed.

- 2. Label a 96-well PCR microplate with date and experiment numbers. Mark the middle of the microplate for better visualization of the positions. Optionally, indicate separations between zones with different multiplexes as shown in Figure 2.
- 3. Dispense 18 μ l of the PCR premix into each well of the microplate.

Figure 2 Delineation of different multiplex zones on PCR microplate



Table 1 Volumes (µl) for the discriminatory multiplex premixes

mix	1	2	3	4	5
Loci	4-26-40	10-16-31	0424- 0577- 2165	2401-3690- 4156	2163b- 1955- 4052
MgCl ₂ final concentration	3mM	2mM	1,5 mM	3mM	1,5 mM
H2O	7,5	8,3	8,7	7,5	8,7
Buffer 10 X	2	2	2	2	2
Q Solution 5x	4	4	4	4	4
MgCl ₂ 25 mM	1,2	0,4	0	1,2	0
DNTP 5mM	0,8	0,8	0,8	0,8	0,8
Primers EACH ^a	0,4	0,4	0,4	0,4	0,4
Hotstart DNA pol	0,08	0,08	0,08	0,08	0,08
Total premix	18	18	18	18	18

^a Six in total, *i.e.* one forward and one reverse primer for each of the 3 pairs. Initial concentration for all unlabeled primers: 20 pmol/ μ l. Initial concentration for the labeled oligonucleotides: 2 pmol/ μ l for locus 0577, 3690 and 1955, 8 pmol/ μ l for locus 4052, 20 pmol/ μ l for locus 4156 and 4 pmol/ μ l for the other loci. See appendix for the primer sequence and labeling.

Note: the Qiagen PCR Multiplex PCR kit may be specifically used for mix 5, to reduce pronounced stutter peaks seen with large alleles of locus 4052 (see Annex 5).

mix	6	7	8
Loci	2-23-39	20-24-27	2347-2461- 3171
MgCl ₂ final concentration	2.5 mM	1,5 mM	2mM
H2O	7,9	8,7	8,3
Buffer 10 X	2	2	2
Q Solution 5x	4	4	4
MgCl ₂ 25 mM	0.8	0	0,4
DNTP 5mM	0,8	0,8	0,8
Primers EACH ^a	0,4	0,4	0,4
Hotstart DNA pol	0,08	0,08	0,08
Total premix	18	18	18

Table 2 Volumes (µl) for the other multiplex premixes

^a Six in total, *i.e.* one forward and one reverse primer for each of the 3 pairs. Initial concentration for all unlabeled primers: 20 pmol/ μ l. Initial concentration for all labeled oligonucleotides: 4 pmol/ μ l. See appendix for the primer sequence and labeling.

Loci	4-26-40- 2401-3690- 4156	10-16-31- 2347-2461- 3171	20-24-27- 0424- 0577- 2165-2163b- 1955- 4052	2-23-39
MgCl ₂ final concentration	3mM	2mM	1,5 mM	2.5 mM
H2O	9,1	9,9	10,3	9,5
Buffer 10 X	2	2	2	2
Q Solution 5x	4	4	4	4
MgCl ₂ 25 mM	1,2	0,4	0	0.8
DNTP 5mM	0,8	0,8	0,8	0,8
Reverse primer ^a	0,4	0,4	0,4	0,4
Forward primer ^a	0,4	0,4	0,4	0,4
Hotstart DNA pol	0,08	0,08	0,08	0,08
Total premix	18	18	18	18

Table 3 Volumes (µl) for the simplex PCR premixes

^a See tables above for the initial concentration. When analysis of the PCR products is performed using only agarose gel electrophoresis (manual typing), all primers are unlabeled.

2.2.2.3 Addition of genomic DNA

- In another PCR product-free area, dispense 2 μl of extracted DNA previously diluted 1:50 into each well. If purified DNA is used, dispense 2 μl of solution at 1 ng/μl. Include H37Rv DNA and sterile water as positive and negative controls, respectively.
- 2. <u>**Tightly**</u> seal the PCR microplate using an adhesive PCR film, to avoid evaporation during amplification.

2.2.2.4 PCR amplification

- 1. Amplify the target loci using the PCR conditions indicated in Table 4. These conditions have been successfully tested on Hybaid PCR express and Perkin Elmer cyclers.
- 2. For analysis on automated sequencers, notice that the number of cycles may need to be adjusted depending on the DNA concentrations routinely obtained in the laboratory, i.e. amplification from weakly or highly concentrated mycobacterial cultures may require 40 cycles or only 25 (Mix 1, 2, 4, 6, 7, 8) to 30 (Mix 3, 5) cycles, respectively.
- 3. Store the PCR products at 4°C or -20°C until further use.

95°C
94°C
59°C
72°C
72°C
4°C

Table 4 PCR cycling conditions

Cycle numbers: - Automated typing: 30 for Mix 1, 2, 4, 6, 7, 8 35 for Mix 3 and 5 - Manual typing: 40 cycles for all mixes

2.3 PCR product analysis using agarose gel electrophoresis

When each locus is amplified separately (i.e. by simplex PCR), the amplified fragments can be analyzed by electrophoresis using agarose gels. This method is inexpensive and easy, as it only requires a size resolution of about 50 bp (except for locus 4, see below). It is accurate, provided that adequate electrophoresis conditions and controls are used. It is suitable for laboratories with relatively low turnovers of isolates to be analyzed. The use of multi-channel pipettes compatible with gel combs is useful for both the speed and the reliability of the genotyping process.

This method is also used to size PCR fragments from reference strains, (in addition to H37Rv), selected to sample the allelic range of each MIRU-VNTR locus. When labeled with fluorescent dyes, the corresponding reference PCR products can then be used to calibrate the sizing using electrophoresis with your DNA sequencer.

The quality of the DNA fragment resolution and the use of a control for possible migration smiling effects are critical for the sizing accuracy. Therefore, long gels and sufficient electrophoresis time should be used. The use of Nu-Sieve agarose gels offering high resolution for small DNA fragments is recommended, especially for the detection of variant alleles in locus 4. However, electrophoresis grade agaroses such as Ultra Pure Electrophoresis Grade Agarose from Gibco-BRL can yield satisfactory results.

2.3.1 Gel preparation and electrophoresis

- Prepare a 3 % suspension of Nu-Sieve agarose gel in 1 x TBE solution previously chilled at 4°C, to facilitate clump collapse.
- 2. Melt the agarose using a microwave oven, and agitate periodically until complete dissolution.
- 3. When the temperature is endurable to the touch, cast a 25-cm gel, using a shark tooth comb.
- After solidification, place the gel into an electrophoresis tank containing 1 X TBE.

- 5. Load 10 μl of a 100-bp ladder size standard marker <u>in both external wells</u> <u>and in the central well</u> of the gel. The standard in central position can be used to detect possible migration smiling effects. A 50-bp ladder or a 20-bp ladder (however less easy to read sometimes) can be used in addition.
- 6. For each reaction, load, per well, a mixture of 2 ml of PCR product with 2 ml of loading buffer. <u>Migrate amplicons from a given locus together on a same gel</u>, rather than migrating different loci from a same isolate on a same gel. By this way, allelic assignation is facilitated by visualization of the band ladders generated by different repeat numbers (see 2.3.2).
- 7. Run at 120 V for 5 hours.
- Stain the gel in sterile water containing 0.7 μg/μl of ethidium bromide for 15-30 min.
- 9. Expose the gel to UV light and take a photo.

2.3.2 Sizing and allele assignation

Most often, amplification results in single sharp PCR products (Figure 3). Depending on the locus, this sharp amplicon can be accompanied by a ladder of much lower intensity bands, called stutter peaks (see 2.3.3 and Figure 4). However, some problematic amplification of loci with large repeat numbers can result in a ladder of bands, with no clearly sharpest band or with a "bell-shaped" distribution of band intensities (see 2.3.3). <u>In this case, do not assign any result and re-amplify.</u>

- 1. Determine the size of the sharp PCR product by comparison with the position of the size standard marker.
- 2. Determine the corresponding repeat number, using the provided table containing the allele calling for each VNTR locus, as shown in Figure 3.

Verify the consistence with the usual allelic range.

- 3. Verify that the allele assignation of the H37Rv control is correct.
- 4. Verify the consistence of the results by judging the incremental spacing between PCR products from different isolates (*i.e.* co-migrating fragments =

same alleles, fragments smaller by one repeat increment = allele-1, etc). This control is best done starting from the smallest amplicon, as sizing is usually more accurate for small products.

5. If classical stutter peak ladders (see 2.3.3 and Figure 4) are present, they can also be used as internal sizing controls to verify the consistency of the allele assignation. Similarly, this control is best done starting from the smallest stutter peak.

354

408

462

516

570

624

678

732

MIRU 40 Allele 700 0 pb 600 1 pb 2 500 3 pb 4 400 5 pb 6 7

Figure 3 Example of allele identification for MIRU-VNTR locus 40

2.3.3 Detection and interpretation of stutter peaks

Stutter peaks are common during genotyping of tandem repeat sequences, and mostly reflect artifactual strand slippage of the polymerase during PCR. Such stutter peaks are also quite frequently observed for PCRs of various MIRU-VNTR loci, more often when containing large repeat numbers.

In most cases, they can be easily diagnosed, as they appear as a ladder of much lower intensity peaks, corresponding to sizes of PCR fragments that lack one or more repeats, or more rarely that contain one or more additional repeat. The positions of stutter peaks can be used to confirm the allelic assignation of the principle fragment (see point 5 in 2.3.2).

As indicated above, sub-optimal amplification of loci with large repeat numbers (such as 4052, alias QUB 26) can sometimes result in a band ladder with no clearly sharpest band or with a "bell-shaped" distribution of band intensities (see next section). This can be typically seen when the

Q buffer is not used. <u>In this case, do not assign any result and re-amplify. Optimal</u> <u>amplification will often result in a single sharp band, with a highest size than could perhaps</u> <u>have been anticipated based on the initial band ladder pattern.</u>

Figure 4 Example of stutter peak detection in a MIRU-VNTR

The example shown corresponds to amplicons from MIRU-VNTR locus 27. Dotted arrows show positions of stutter peaks. The size increments between the stutter peaks correspond to the size of one repeat unit (53 bp in this case). The size standard (M) is a 20-bp ladder.

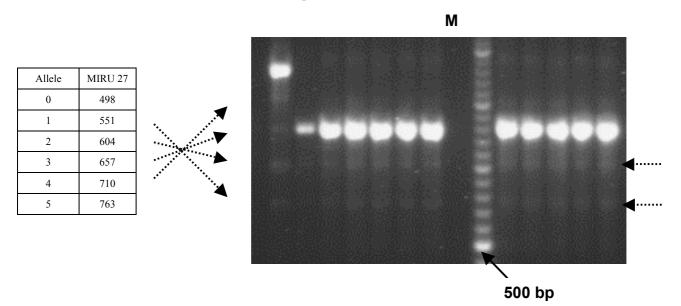
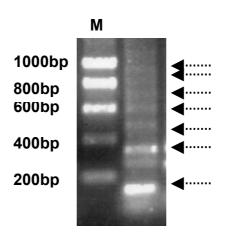


Figure 5 Example of "bell-shaped" ladder

The example shown corresponds to fragments amplified from MIRU-VNTR locus 4052 (alias QUB-26). Arrows show positions of stutter peaks. The size increments between the stutter peaks correspond to the size of one repeat unit (111 bp in this case). M, size standard (M).



2.4 PCR product analysis using capillary electrophoresis

2.4.1 Principle

The PCR fragments labeled with the three different fluorescent dyes from each multiplex are combined with an internal size standard, labeled by a fourth dye and then analyzed in individual capillaries on a DNA sequencer for size determination.

2.4.2 Capillary electrophoresis

The procedure is described for ABI 3100 sequencers, using the Data Collection software version 1.1. It is applicable to 3100 Avant and/or to software versions 2.x with minimal adaptations. Some additional adaptations may be needed for 3130, and 3700 and/or other software versions.

A summary flowchart of a typical sequencer run is displayed in Figure 6. Operations specific to MIRU-VNTR typing are described starting from sample preparation (see arrow in Figure 6). Information for spectral calibration for the current dye set used in MIRU-VNTR typing is given in 2.4.2.1.

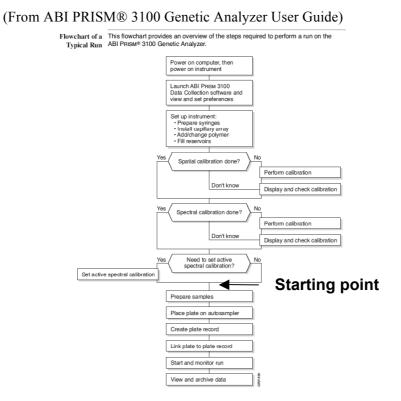


Figure 6 Summary flowchart of a typical sequencer run

2.4.2.1 Dye set and spectral calibration

If not done yet for other types of fragment analysis, initial spectral calibration is required for the appropriate detection of the current dye set used in MIRU-VNTR analysis. This combination of dyes (6FAMTM, VIC®, NEDTM, for the amplicons, and ROXTM for the size standard) corresponds to dye set D on ABI sequencers.

This spectral calibration is performed using ABI DS-31 Matrix Standard Kit, containing 4 oligonucleotides respectively labeled with these 4 dyes, pre-pooled in a single tube. Spectral calibration procedure is described in the instructions for use and in the sequencer user guide.

2.4.2.2 Preparing the samples

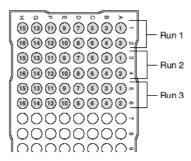
Information on how samples are scheduled for injection based on plate configuration is provided in Figure 7.

Figure 7 96-well plate mapping

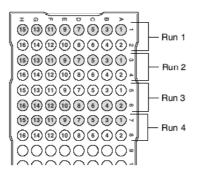
(From ABI PRISM® 3100 Genetic Analyzer User Guide)

96-Well Plate For a 96-well plate, injections are made from every well in two consecutive rows, Mapping starting with an odd row. A full 96-well plate requires six runs to inject all samples.

Below is an example of a 96-well plate. The gray circles represent samples, and the number in the well indicates capillary number. It takes three runs to inject 48 samples.



Below is an example of incorrect sample placement. To inject 24 samples requires four runs.



The sample are prepared as follows:

- Dispense 1 µl of PCR product into the wells of a plate adapted for the ABI sequencer (e.g. Semi-skirted PCR plates, Sorenson, BioScience). Verify that each well contains solution.
- Prepare extemporaneously a mix of 10 µl of formamide (Applied Biosystems) and 0.2 µl of MapMarker 1000 ROX[™] size standard (BioVentures), per sample.
- Dispense 10 μl of this mix into the wells of the plate. Verify that each well contains solution.
- 4. Tap down or centrifuge the plate so that each sample is positioned at the bottom of its well. Air injection should be avoided. Therefore your sample should:

Look like this	Not look like this	Not look like this
CERT300		
The sample is positioned correctly in the bottom of the well.	The sample lies on the side wall because the plate was not centrifuged.	An air bubble lies at the bottom of the well because the plate was not centrifuged with enough force or time.

(From ABI PRISM® 3100 Genetic Analyzer User Guide)

5. Keep the plate at 4°C until you are ready to prepare the plate assembly and place the assembly on the autosampler. (*note*: such DNA/formamide mixtures can be kept for a few days, but no longer than one week at 4°C).

2.4.2.3 Editing or creating a specific run module

Create a specific run module, called GS36_POP4_3000sec, as decribed in Figure 8, according to parameters in Table 5.

Figure 8 Creating a specific module

(From ABI PRISM® 3100 Genetic Analyzer User Guide)

Step	Action						
1	Click the Module Editor button on the toolbar to open the Module Editor dialog box.						
	िद्रि Select						
2	Select a run module to use as a template.						
3	Edit the parameter values that you want to change.						
	IMPORTANT Only whole numbers are accepted.						
	IMPORTANT Be sure that all values are red. Values in black are not saved.						
4	Click Save As to create a new run module.						
	Enter a unique descriptive name and click OK.						
	Enter Norte of New Mockie: Try_new_module OK Cancel Enter GS36_POP4_3000sec						
	Note Save cannot be applied to default run modules.						
5	When you are finished, click the Close button (<u>×</u>) to exit the Module Editor.						

Table 5 Run module parameters for ABI 3100, 3100 Avant

Run Temperature:	60 degrees
Cap Fill Volume:	184 steps
Current Tolerance:	100 micro Amps
Run Current:	100 micro Amps
Voltage Tolerance:	0.6 kVolts
Pre Run Voltage:	15 kVolts
Pre Run Time:	180 sec
Injection Voltage:	1 kVolts
Injection Time:	22 sec
Run Voltage:	15 kVolts
Number of Steps:	10 nk
Voltage Step Interval:	60 sec
Data Delay Time:	1 sec
Run Time:	3000 sec
1, 1,	

2.4.2.4 Creating a plate record template

Plate records (or sample sheets) are data tables that store information about the plates and the samples they contain. Specifically for fragment analysis, a plate record contains the following information:

◆ Plate name, type, and owner

- Position of the sample on the plate (well number)
- ♦ Sample name
- Dye color of size standard
- Comments about the plate and about individual samples
- ♦ Dye set information
- ◆ Project name (this entry is mandatory, even when Sequence Collector is not used)
- Name of the run module (run modules specify information about how samples are run)
- ♦ Name of the analysis module (analysis modules specify how raw data is

auto-analyzed at the end of the run). Auto-analysis is usually not performed.

Plate records can be generated using the Data Collection software, by following the steps in Figure 9. Specific notes for MIRU-VNTR analysis are indicated in bold. **Do not forget to fill in color info with sample name for each color.** This information is needed to create tables with final results in Genotyper templates.

Alternatively, plate records can be conveniently created using Excel and then subsequently imported as tab files (see next section).

Figure 9 Creating a plate record for fragment analysis

(From ABI PRISM® 3100 Genetic Analyzer User Guide)

To enter plate record information:

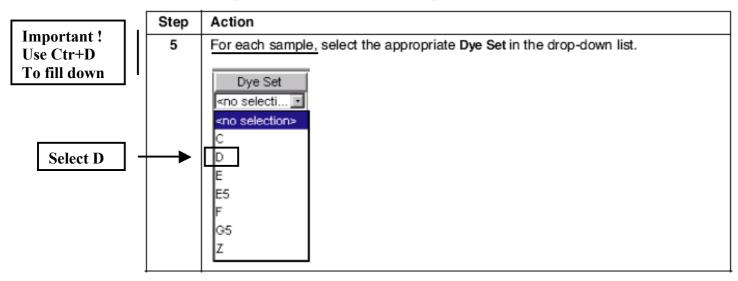
Action
Click the Plate View tab in the data collection software window to go to the Plate View page.
Plate View Run View Status View Array View Capillary View
In the Plate View page, click New.
The Plate Editor dialog box opens.
Enter your plate name and select the application and plate type. Comments are optional.
Plate Editor Image: Second
When done, click Finish. The plate editor spreadsheet opens.
New Y Color Connent Project Name Dye Set Run Notuble Viel Sample Name Dyes Color Info Color Connent Project Name Dye Set Run Notuble A1 B 0 0 0 0 0 0 0 0 0 0

Figure 9 (continued)

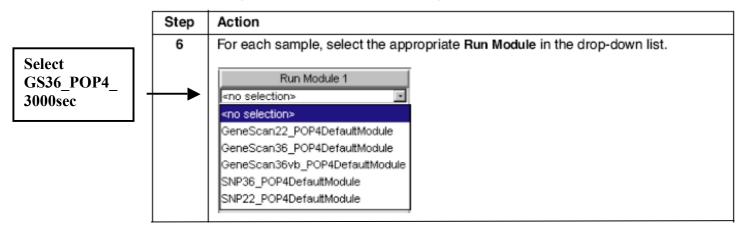
Entering Sample	To enter sample information and save the plate record:			
Information	Step	Action		
	1	In the Plate Editor spreadsheet, type the names of all the samples in the Sample Name column. Sample names are limited to a maximum of 32 characters.		
Important !		 When naming the samples, you can use letters, numbers, and the following punctuation only:(){#.+. DO NOT USE SPACES. 		
		 In the default naming convention, the sample name you type is incorporated into the sample file name. For example: 		
		PlateName_A01_MySample01.fsa Fragment analysis file extension Capillary position Sample name you type		
		Well position		
		Plate name you type		
		· ·		
		Note The sample file naming convention used can be changed in the Preferences dialog box. See page 2-12 for details.		
Not applicable	2	Change the size standard dye color, if necessary. The default is red. Use red for 4-dye applications and orange for 5-dye applications.		
Size standard color		a. Click on the dye color you want to use.		
is red (ROX)		b. Select all the samples.		
		c. Select Edit > Fill down.		
	3	Type in Color info and Color Comment, if needed.		
		Note Color Info and Color Comment information is the same as Sample Info and Sample Comment in the ABI PRISM® Genotyper® software. Refer to the ABI PRISM® Genotyper® Software User's Manual for more information.		
	4	Enter a Project name.		
Important !		Note A project name is required for every sample, even if a Sequence Collector database is not used.		
		a. Click in the Project Name cell for Well A1.		
		b. Select a project name in the drop-down list.		
		Project Name <no selection=""> <no selection=""> <no selection=""> 3100_Project1 Note You must select a project.</no></no></no>		
		c. To assign the same project name to each sample in the plate record:		
		 Click the column header to select the whole column. 		
		– Press Ctrl+D.		
		Note Press Ctrl+D to fill down whenever a field is the same for all samples in the		
		plate record.		

Figure 9 (continued)

To enter sample information and save the plate record: (continued)



To enter sample information and save the plate record: (continued)



To enter sample information and save the plate record: (continued)

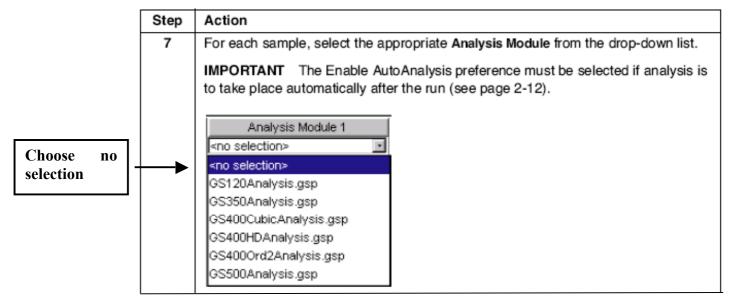


Figure 9 (continued)

	8	Ru Ru Note run r	in Module 2 Sample module are erify that th	s mo es wil e run e pla	adule. ' Analysi: Il be al seque ate rec	You can s Module utomatic entially. ord is co	run a san 2 ally group prrect and	pple in bed so	that all samp	module and a up to five times les with the san the following or, project, and	ne
		b. Cl An ex Plate E	odule). ick OK. kample of a ditor tat		nplete	d plate r	ecord is s	hown	below.	Ē	×
			Well Sample Name A1 Sample1	Dyes B G Y R O D	Sample 1 Sample 1 Sample 1 Sample 1 Sample 1 Sample 1 Sample 2	Color Comment	Project Name 3100_Project1 3100_Project1	Oye Set	Run Module 1 GeneScan36_POP4Defa.		
Fill co	<u>ith</u>		bi Saliper	o Y ▼	Sample 2 Sample 2 Sample 2 Sample 2 Sample 2						
for each color	<u>e name</u> c <u>h</u>		C1 Sample3	B G Y R	Sample 2 Sample 3 Sample 3 Sample 3 Sample 3 Sample 3		3100_Project1	65	GeneScan36_POP4Defa.	. GS500Analysis.gap	
But no applic			D1 Sample4	D V R	Sample 4 Sample 4 Sample 4 Sample 4 Sample 4		0100_Project1	85	GeneScar00_POP4Defa.	. 63500Analysis ysp	
for Da	<u>ita</u>		El SampleS	B G Y R	Sample 5 Sample 5 Sample 5		3100_Project1	G5	GeneScan36_POP4Defa.	GSS00Anelysie.gep	
<u>versio</u> <u>Must</u>	<u>be</u>	(Commente:								
<u>entere</u> <u>Genot</u> <u>See 2.0</u>	vper		base and a	ddeo	d to the	e Pendin	ig Plate R	•	e record to be s table as show		
		THE OWNER OF T	00 Data Col View Instrur								
		Plate	View Run V	/iew	Status V		y View Cap	illary ∨i	ew		
			Pending Plate	Recor	ds						
			Plate Nan my_plate	ne	App GS	lication	VVells 96	q	Status ending		

2.4.2.5 Importing plate record tab files generated with Excel

- Create an Excel table following the model shown in Figure 10. Type sample names in cells B4 to B483. <u>Add extensions to sample names, if different multiplex or</u> <u>loci are used in a same plate (*e.g.* -M1 for Mix 1, -04 for locus 4). Do not use spaces, or punctuation incompatible with PC file name formats.
 </u>
- Select the sample name cells, Copy (Ctrl+C) and Paste (Ctrl+V) these cells into cells F4 to F483. This will be useful to retrieve samples in Genotyper.
- 3. For positions containing no samples, type a 0 into all corresponding sample name cells for the five colors.

	(💧 Exce	I Fichier	Edition Aff	fichage Ins	ertion	Format	Outils Dor	nées Fen	être Aide		
	0	la 🗊 🖻) 🌾 🖨 🛛	🔍 🔏 🐚 🛛	🛍 🔮 🔝	• 🔊 •	$\sum \cdot f_{2}$: 👷 🥋 💷		00% 🔻 💦 🕨		
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		H4	▼ :	× 🗸 📓	3130Pro	oject1						
	0	00				/		samplesheet	model.txt			
	\diamond	A	B	С	D	E	F	G	н		J	K
	1	1.0										
	->	run01Phil	GS	96-Well	Test3130							
	3	Well	Sample Name	Color Number	Standard Dye	Dye Set	Color Info	Color Comment	Project Name	Sample Tracking Id	Run Module	Analysis Module
	4	A1	409-03	1	4	D	409-03		3130Project1		GS36_POP4_3000	
		A1	409-03	2	4	D	409-03		3130Project1		GS36_POP4_3000	
		A1	409-03	3	4	D	409-03		3130Project1		GS36_POP4_3000	
		A1	409-03	4	4	D	409-03		3130Project1		GS36_POP4_3000	
	8	A1	409-03	5	4	D	409-03		3130Project1		GS36_POP4_3000	
		B1	192-02	1	4	D	192-02		3130Project1		GS36_POP4_3000	
	10	B1	192-02	2	4	D	192-02		3130Project1		GS36_POP4_3000	
	11	B1	192-02	3	4	D	192-02		3130Project1		GS36 POP4 3000	
	12	B1	192-02	4	4	D	192-02		3130Project1		GS36 POP4 3000	
	13	B1	192-02	5	4	D	192-02		3130Project1		GS36_POP4_3000	
	13 14 15 16	C1	1091-02	1	4	D	1091-02		3130Project1		GS36 POP4 3000	
	15	C1	1091-02	2	4	D	1091-02		3130Project1		GS36 POP4 3000	
	16	C1	1091-02	3	4	D	1091-02		3130Project1		GS36 POP4 3000	
	17	C1	1091-02	4	4	D	1091-02		3130Project1		GS36_POP4_3000	
	18	C1	1091-02	5	4	D	1091-02		3130Project1		GS36 POP4 3000	
	19	D1	1274-03	1	4	D	1274-03		3130Project1		GS36 POP4 3000	
	20	D1	1274-03	2	4	D	1274-03		3130Project1		GS36_POP4_3000	
	21	D1	1274-03	3	4	D	1274-03		3130Project1		GS36 POP4 3000	
	22	D1	1274-03	4	4	D	1274-03		3130Project1		GS36 POP4 3000	
	23	D1	1274-03	5	4	D	1274-03		3130Project1		GS36_POP4_3000	
	24	E1	1275-03	1	4	D	1275-03		3130Project1		GS36 POP4 3000	
	25	E1	1275-03	2	4	D	1275-03		3130Project1		GS36_POP4_3000	
	26	E1	1275-03	3	4	D	1275-03		3130Project1		GS36 POP4 3000	
	27	E1	1275-03	4	4	D	1275-03		3130Project1		GS36_POP4_3000	
	22	E1	1275-03	5	4	D	1275-03		3130Project1		GS36_POP4_3000	
	19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34	F1	900-02	1	4	D	900-02		3130Project1		GS36_POP4_3000	
	29	F1	900-02	2	4	D	900-02		3130Project1		GS36_POP4_3000	
	30	F1	900-02	2	4	D	900-02		3130Project1		GS36_POP4_3000 GS36_POP4_3000	
	31	F1 F1	900-02	4	4	D	900-02				GS36_POP4_3000 GS36_POP4_3000	
	32	F1 F1	900-02	4	4	D			3130Project1			
	33	G1	1501-02	5	4	D	900-02 1501-03		3130Project1 3130Project1		GS36_POP4_3000 GS36_POP4_3000	
	- 34	61	1501-03	1	4	D	1501-03	1	3130Protect1		G530 P0P4 3000	

Figure 10 Plate record model using Excel

- 4. Save it as a tab-delineated file (.txt).
- In Data Collection Software, click Plate View tab, then click Import (see Figure 11).
- 6. Select all types (*.*) from files of type.
- 7. Select your plate and click OK.

- 8. Double click on the pending plate and verify that the record is correct and complete (see example in step 9 of Figure 9).
- 9. If positions contain no sample (now indicated by a 0 after plate record importation), click on the **Run Module** cell of the first empty well and select **No Run Module**. Select the first and last empty positions by pressing **Shift** key, and then press **Ctrl+D** to fill down.

Figure 11 Plate record importation and checking

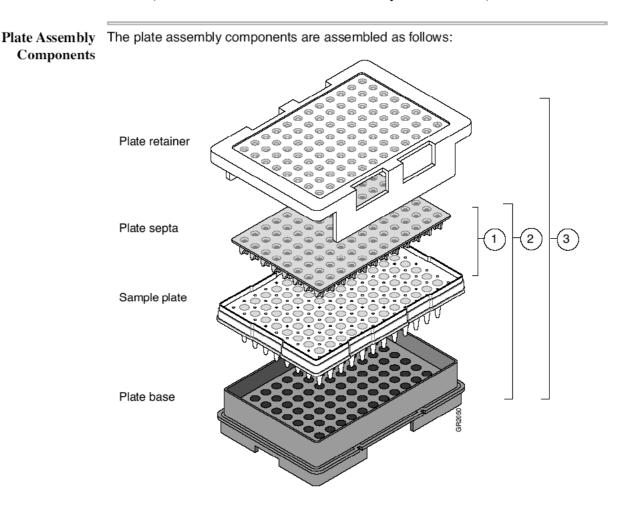
(From ABI PRISM® 3100 Genetic Analyzer User Guide)

my_record Se se pending inked Plate Records Plate Name Application Wells Status Processed Plate Records Plate Name Application Wells Status	Plate Name my_plate	Application GS	Vièls 96	Status pending
Plate Name Application Wells Status	my_record	58	96	pending
Prace warne Application Vveits Status		4	1 100.00	Chabura
	Plate Name	Application	VVeis	Status

2.4.2.6 Preparing and placing a plate assembly

<u>Verify the absence of any air bubble in plate wells.</u> If needed, centrifuge the plate to remove them. Prepare a plate assembly and place it on the Autosampler by carefully following the steps described in Figure 12.

Figure 12 Plate assembly and placing onto the Autosampler



(From ABI PRISM® 3100 Genetic Analyzer User Guide)

eparing a Plate Assembly	To prepa	re a plate assembly:			
Assembly	Step	Action			
	1	Secure a clean and dry plate septa on the sample plate.			
		IMPORTANT Never use warped plates.			
	IMPORTANT Ensure that the plate septa lies flat on the plate.				
	2	Place the sample plate into the plate base.			
	-				

Step	Action						
3	Snap the plate retainer onto the plate and plate base.						
4	Ensure that the plate retainer holes are aligned with the holes in the septa strip.						
	IMPORTANT Damage to the array tips will occur if the plate retainer and septa strip holes do not align correctly.						
	The plate retainer holes in the plate septa.						

Placing the Plate To place the plate onto the autosampler:

onto the	·						
Autosampler	Step	Action					
· · · · · · · · · · · · · · · · · · ·	1	Place the plate assembly on the autosampler in position A or B.					
		Note There is only one orientation for the plate, with the notched end of the plate base away from you.					
		Plate position B					
		Plate position A					
		IMPORTANT Ensure the plate assembly fits flat in the autosampler. Failure to do so may allow the capillary tips to lift the plate assembly off of the autosampler.					
	2	When the plate is correctly positioned, the plate position indicator on the Plate View page changes from gray to yellow.					
		Verify that this has happened.					
	3	Close the instrument doors.					
		Note Closing the doors returns the autosampler to the home position, placing the tips of the capillaries in buffer.					

2.4.2.7 Linking a plate

A link must be created between the plate on the auto-sampler and the corresponding plate record, before a run can be performed. This procedure and the verification of the run schedule are described in Figure 13.

(Fro	om ABI	PRISM® 3100 Genetic Analyzer User Guide)	
Linking a Plate to a		plate to a plate record:	
Plate Record	Step	Action	
	1	Click the Plate View tab.	—— Plate View tab
	2	In the Plate View page:	
	2	a. In the Pending Plate Records table, click the plate record for linking.	
		b. Click the plate position indicator that corresponds to the plate	you are linking.
		Pending Hitle Records Pidde Name Application Note: In the plate in Pidde Name Application Pidde Name Application Click the plate in Pidde Name Application	record
		Processed Flate Resords Proces	
		New	
		Does the following message display?	Then proceed to step
		One or more plate wells are missing information, and will be skipped. Please check the Status View for more detailed information. OK	
		Yes	3
		No	4
	3	Add the required information to the plate record:	
		 Unlink the plate record, if necessary. (The plate record return Plate Records table.) 	is to the Pending
		b. Double-click the plate record name to open it.	
		c. Correct the plate record and click OK .	
		d. Link the plate record to the plate again.	

Figure 13 Linking a plate and viewing the run schedule

To link a plate to a plate record: (continued)

Step	Action
4	Verify that the plate has been linked.
	Once the plate has been linked, the:
	• Run Instrument button in the toolbar is enabled, meaning that the instrument is ready to run.
	 Plate position indicator for the linked plate becomes green.
	 Plate record moves from the Pending Plate Records table to the Linked Plate Records table.
	Run Instrument Plate position button is enabled indicator is green
	1100 Date Collection Software - Version 1.1
	Prote Year Particip Partic
	Plate record is in the Linked Plate Records table

Viewing the Run After a plate is linked, use the Run View page to verify that runs are scheduled Schedule correctly.

To view the scheduled runs:

Step	Action
1	Click the Run View tab. Run View Status View Array View Capillary View
2	Select a row for any run. The corresponding wells to be injected for that run becomes highlighted in the plate diagram on the left. For more information about the Run View page, see page 2-62.

Note Although you can delete individual runs, you cannot alter the order in which the runs are scheduled.

2.4.2.8 Launching and controlling the run

The procedure for starting and controlling a run is displayed in Figure 14.

Figure 14 Launching and controlling a run

(From ABI PRISM® 3100 Genetic Analyzer User Guide)

Starting the Run To start a run:

Step	Action							
1	If you want to review the run schedule before beginning the run, click the Run View tab.							
2	File View Instrum							
3	The software automatically checks the available space in the database and drive D. If the database or drive D are							
	full	 a. proceed to "Automatic Checking of Available Space on Drives D and E" on page 2-55. b. Make more space. c. Click Run Instrument. 						
	not full	the run starts.						

Controlling the Run Using the Toolbar Vou can also use the toolbar at the top of the data collection software window to control the run.



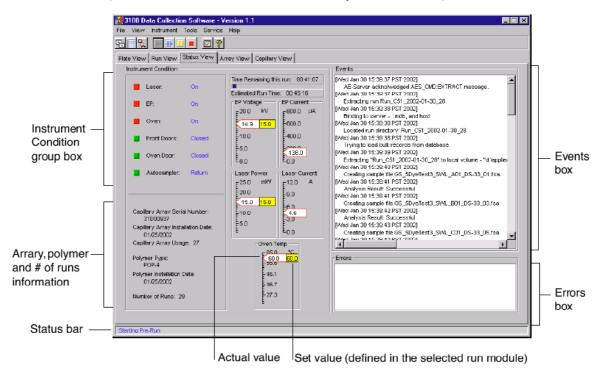
То	Click	Comment
Start the run	Run Instrument	 This begins all scheduled runs. The run starts only when set temperature is reached.
Pause the run	Pause	Pausing the instrument for too long, especially after sample injection, will affect data quality. The best time to pause is before sample injection
 Complete the current run, and 	a. Stop	
 Stop the other scheduled runs 	b. After run in the Question dialog box	
 Stop the current run, and Stop the other scheduled runs 	a. Stop	When you click Now , the run files extract automatically. The files will be automatically analyzed if the AutoAnalysis preference is enabled.
	Stop now or after current run? Now	To recover data from a stopped run, see "Recovering Data If Autoextraction Fails" on page 2-70.
 Stop the current run, and Continue the other scheduled runs 	Skip to Next Run	To recover data from a stopped run, see "Recovering Data If Autoextraction Fails" on page 2-70.

2.4.2.9 Monitoring the run status

The run status can be monitored by clicking the Status View tab. An example of Status view page is provided in Figure 15.

Figure 15 Example of Status View page

(From ABI PRISM® 3100 Genetic Analyzer User Guide)



2.5 PCR product sizing using Genescan

After electrophoresis, the sizes of the PCR fragments are estimated using the GeneScan Analysis software (Applied Biosystem). Size data editing and automated conversion of these data into MIRU-VNTR alleles is only done subsequently, using customized templates of the Genotyper software (see next section). The procedure described below is based on GeneScan version 3.7.1.

IMPORTANT: this version must be initially updated with GeneScan version 3.7.1 Updater and switched to Large Fragment Analysis using <u>Large Fragment Enabler</u>, as indicated in the ReadMe instructions included the Updater CD.

2.5.1 Principle

The GeneScan Analysis software is used to calculate the apparent sizes of the PCR products, by correlating their migration data (in terms of laser scan numbers needed for detection in the CCD window) to those of size standard bands.

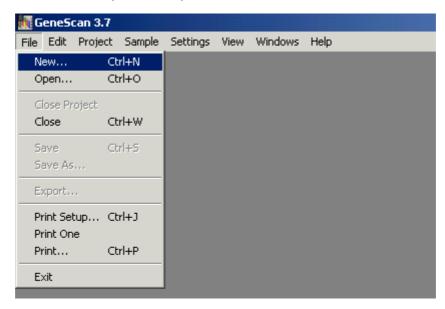
2.5.2 Procedure

2.5.2.1 Creating a GeneScan project

1. Open Genescan

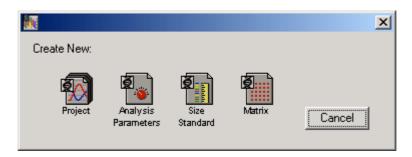


2. From the main menu, click **File**, then **New**.

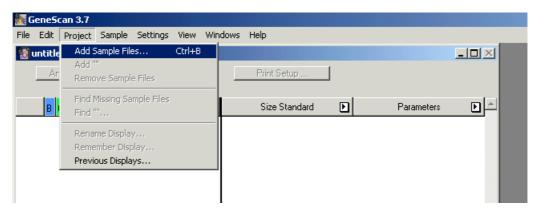


3. Click Project on the window as displayed below, to open the Analysis Control

Window.



4. Click Project from the main menu, and then Add Sample Files.

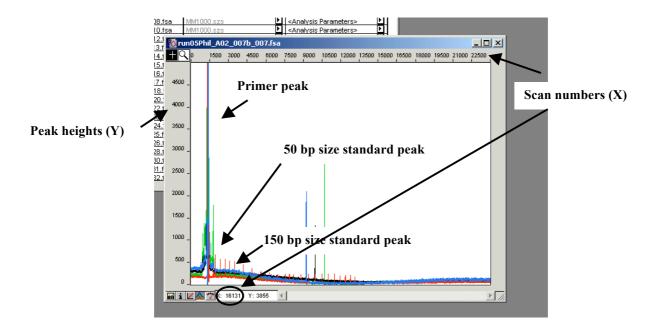


5. Select the folder containing the sample files, and then click Add All, then Finish.

W GeneScan 3.7 File Edit Project Sample Settings View Win	dows Help	
🔝 untitled 2 - Analysis Control	-D×	
Analyze Print Results	Print Setup	
B G Y R Sample File	Size Standard D Parameters	
	Add Sample Files	<u>?</u> ×
	Rechercher dans : 🛛 🔁 Run_3700_2005-03-30_50 🛛 🔽 🖨 📸 🎬 🕶	
	Image: Second	AF2
	Remove Remove All Finish Uancel	

2.5.2.2 Setting analysis parameters and size standard reference

- Double click on a Sample File and then click A at the bottom of the pop-up window, to visualize an electropherogram which should look like the example below.
- 7. Using the zoom and the pointer A, visualize and notice the scan numbers (X) corresponding to the zone immediately after the primer peak, and to the end of the run, respectively. This range should include all size standard peaks (in red) starting from 50 bp (see below for the size standard definition).



 Close the electropherogram window. Double-click any Analysis Parameters cell, to open the Analysis Parameters window.

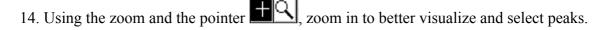
- 9. Define the start and stop of the analysis range by introducing the scan numbers corresponding to the zone comprised <u>between the primer peak and the 50 bp</u> <u>peak</u>, and <u>just before to the end of the run</u>, respectively. Define the other parameters as shown below.
- 10. Save as Analysis Parameters. This file will then be used as default.
- 11. Note: Peak amplitude thresholds, defining the minimum peak height detection, can be subsequently increased if higher backgrounds are noticed for some samples. In this case, save the modified analysis parameters under a new name.

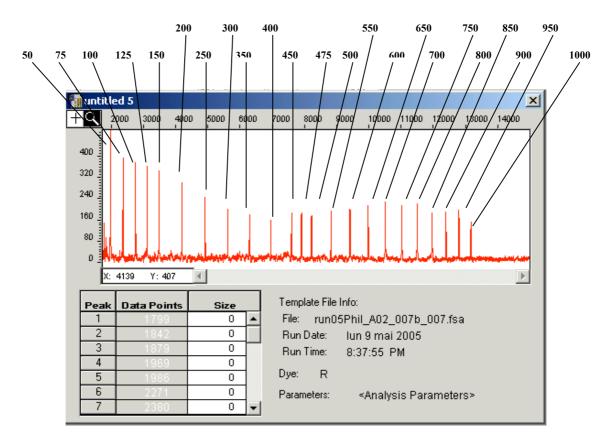
	7				
Example					
values only	Sample File	Size Standard	Ð	Parameters	Ð
· ·	M Analysis Parameters	X	D	<analysis parameters=""></analysis>	Ē
Adapt to	Analysis Range	Size Call Range		<analysis parameters=""></analysis>	Þ
-	C Full Range	 Full Range 		<analysis parameters=""></analysis>	
vour case	This Range (Data Points)	C This Range (Base Pairs)		<analysis parameters=""></analysis>	_ D
-	art: 1650			<analysis parameters=""> <analysis parameters=""></analysis></analysis>	Ē
-		Min: D		<analysis parameters=""> <analysis parameters=""></analysis></analysis>	Ē
-	Stop: 20000	Max: 1000		<analysis parameters=""></analysis>	Ð
	Data Processing	Size Calling Method		<analysis parameters=""></analysis>	Ð
_	Data Hocessing			<analysis parameters=""></analysis>	
-	Smooth Options —	C 2nd Order Least Squares		< <u>Analysis Parameters></u>	<u>ا</u>
-	C None	C 3rd Order Least Squares		<analysis parameters=""></analysis>	- <u>P</u>
-	Light	C Cubic Spline Interpolation		<analysis parameters=""> <analysis parameters=""></analysis></analysis>	Ē
-	C Heavy	Content Southern Method		<analysis parameters=""></analysis>	- F
_	e neavy	C Global Southern Method		<analysis parameters=""></analysis>	Ð
_				<analysis parameters=""></analysis>	
_	Peak Detection	Baselining		<analysis parameters=""></analysis>	
-	Peak Amplitude Thresholds	BaseLine Window Size		< <u>≺Analγsis Parameters></u>	<u>۱</u>
-				<analysis parameters=""> <analysis parameters=""></analysis></analysis>	Ē
-	B: 100 Y: 100	251 Pts		<analysis parameters=""></analysis>	-
-	G: 100 R: 100	Auto Analysis Only		<analysis parameters=""></analysis>	Ē
_		Size Standard:		<analysis parameters=""></analysis>	
_	Min. Peak Half Width: 2 Pts	Size Standard.		<analysis parameters=""></analysis>	
_		<none> 🔻</none>		<analysis parameters=""></analysis>	
-				<analysis parameters=""></analysis>	<u>ا</u>
-	Polynomial Degree 3			< <u>Analysis Parameters></u> <analysis parameters=""></analysis>	Ē
-				<analysis parameters=""></analysis>	- F
-	Peak Window Size 19 Pts			<analysis parameters=""></analysis>	Þ
				<analysis parameters=""></analysis>	Þ
_	Slope Threshold for 0.0			<analysis parameters=""></analysis>	Þ
_	Peak Start			<analysis parameters=""></analysis>	
-	Slope Threshold for			< <u>Analysis Parameters></u>	<u>ا</u>
-	Slope Threshold for 0.0 Peak End			<analysis parameters=""> <analysis parameters=""></analysis></analysis>	- F
-				< <u>Analysis Parameters></u> <analysis parameters=""></analysis>	- 1
-				<analysis parameters=""></analysis>	Ē
-		Cancel OK		<analysis parameters=""></analysis>	▶
				<analγsis parameters=""></analγsis>	
-		WW1000.323		<analysis parameters=""></analysis>	
		MM1000.szs		≺Analvsis Parameters>	

12. Define the size standard reference, by clicking *<None>* as shown below, better from the capillary containing the PCR negative control. This reference can be used for subsequent analysis using the same conditions and run module. If conditions such as voltage or the polymer are changed, this size standard will need to be redefined.

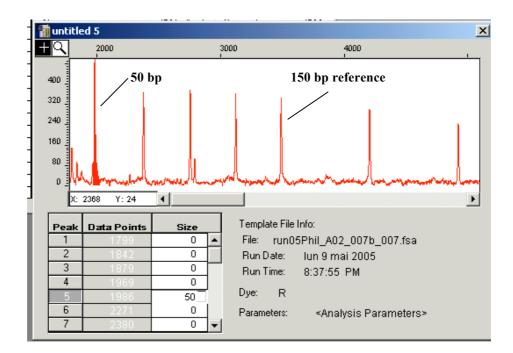
File Edit Project Sample Settings View Windows Help										
🕈 untitled 4 - Analysis Control										
	Ar	aly	ze		Print Results	Print Setup				
	в	a <mark>k</mark>	2 F	2	Sample File	Size Standard	[Ð	Parameters	Þ
1			4	Þ	run05Phil A01 061a 002.fsa	<none></none>	Ū	Ð	<analysis parameters=""></analysis>	Ð
2			4		run05Phil CO1 105b 004.fsa	<none></none>	ļ	Þ	<analysis parameters=""></analysis>	Þ
3			4	>	run05Phil E01 005a 005.fsa	- Define New		Þ	<analysis parameters=""></analysis>	
4			4	*	run05Phil G01 009a 006.fsa	Denne wew		۶.	<analysis parameters=""></analysis>	Þ
5			4	2	run05Phil A02 007b 007.fsa	✓ <none></none>		Z	<analysis parameters=""></analysis>	
6	T		4		run05Phil C02 062b 008.fsa			٢	<analysis parameters=""></analysis>	
7			4		run05Phil B01 105a 010.fsa	A01-RDRio1.szs	1	Þ	<analysis parameters=""></analysis>	Ð
8	T		4		run05Phil D01 104a 012.fsa	CO2-RDRio1.szs		Ľ	<analysis parameters=""></analysis>	Ð
9			4		run05Phil F01 055a 013.fsa	GS 120.szs		Þ	<analysis parameters=""></analysis>	Þ

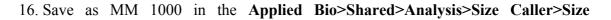
13. The size standard electropherogram corresponding to the selected capillary appears, as indicated below. The sizes of the size standard peaks of the MapMarker 1000 (except the 25 bp peak) are displayed.





15. Enter the sizes corresponding to the data points of each size standard peak, <u>using the</u> <u>150 bp peak as a reference</u>. Carefully verify the correspondence with the general size standard pattern shown above, especially the presence of the typical 450-475-500 bp pattern.





Standard Folder.

Save this document	as	? ×
Enregistrer dans :	🔁 SizeStandards 💿 🗢 🖻 📸 -	
 A01-RDRio1.szs C02-RDRio1.szs G5 120.szs G5 350 377.szs G5 350 All.szs G5 350-250.szs 		
Nom de fichier : Type :	MM 1000 Enregist MM 1000.szs Annuk	_

2.5.2.3 Analyzing sample files

17. Apply this size standard to all samples using the scroll-down menu as shown below.

	В	G	Y	R	Sample File	✓ <none></none>	D	Parameters	
1				۲	run05Phil A01 061a 002.fsa	<collection setting=""></collection>		<a∩alγsis parameters=""></a∩alγsis>	Þ
2				۲	run05Phil C01 105b 004.fsa			<analysis parameters=""></analysis>	
3					run05Phil E01_005a_005.fsa	A01-RDRio1.szs		<analysis parameters=""></analysis>	
4				۲	run05Phil G01_009a_006.fsa	C02-RDRio1.szs		<analysis parameters=""></analysis>	Þ
5				۲	run05Phil A02 007b 007.fsa			<analysis parameters=""></analysis>	Þ
6					run05Phil CO2 062b 008.fsa	GS 120.szs		<analysis parameters=""></analysis>	
- 7				۲	run05Phil B01 105a 010.fsa	GS 350 377.szs		<analysis parameters=""></analysis>	Þ
8				۲	run05Phil D01_104a_012.fsa	GS 350 All.szs		<analysis parameters=""></analysis>	
9				۲	run05Phil F01_055a_013.fsa	GS 350-250.szs		<analysis parameters=""></analysis>	
10				۲	run05Phil H01_007a_014.fsa	GS 400HD.szs		<analysis parameters=""></analysis>	
11					run05Phil B02 062a 015.fsa			<analysis parameters=""></analysis>	
12				۲	run05Phil D02 068a 016.fsa	GS 500 377.szs		<analysis parameters=""></analysis>	
13				۲	run05Phil E02 099a 017.fsa	GS 500 All.szs		<a∩alγsis parameters=""></a∩alγsis>	
14					run05Phil G02 093a 018.fsa	GS 500-250.szs		<analysis parameters=""></analysis>	
15				۲	run05Phil A03 093c 020.fsa	GS2500.szs		<analγsis parameters=""></analγsis>	Þ
16					run05Phil CO3 097b 022.fsa			<analγsis parameters=""></analγsis>	
17					run05Phil E03 097d 023.fsa	GSC01.szs		<analysis parameters=""></analysis>	- 10
18					run05Phil G03 053a 024.fsa	MM 1000.szs		<anal∨sis parameters=""></anal∨sis>	P

18. Click on the zone indicated below to select all sample files for analysis of all fluorochromes, and then click **Analyze**.

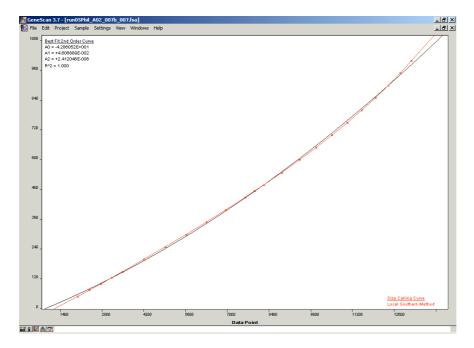
Click to select color analysis	Image: Constraint of Constraints File Edit Project Sample Settings View Windows Help Image: Constraint of Constraints Image: Constraint of Constraints Image: Constraint of Constraints Print Setup	
allaly 515	BGYR Sample File Size Standard	d 🕨 Parameters 🕨 📥
	1 2 run05Phil A01 061a 002.fsa MM 1000.szs	Analysis Parameters>
	2 2 run05Phil C01 105b 004.fsa MM 1000.szs	Analysis Parameters> Analysis Parameters> Analysis Parameters> Analysis Parameters>
	3 _ 🙀 🙀 run05Phil E01 005a 005.fsa 🛛 MM 1000.szs	🛛 <analysis parameters=""></analysis>
	4 🛛 🖉 run05Phil G01 009a 006.fsa 🛛 MM 1000.szs	🕑 «Analysis Parameters» 🕑
	5 🛛 🗖 run05Phil A02 007b 007.fsa MM 1000.szs	Analysis Parameters>
	6 🔽 🖉 run05Phil C02 062b 008.fsa 🛛 MM 1000.szs	🕑 «Analysis Parameters»
	7 2 2 run05Phil B01 105a 010.fsa MM 1000.szs	Analysis Parameters>
	8 2 run05Phil D01 104a 012.fsa MM 1000.szs	🕑 «Analysis Parameters» 🕑
	9 vun 05Phil F01 055a 013.fsa MM 1000.szs	と «Analysis Parameters» 🕑
	10 🔽 🔽 run05Phil H01 007a 014.fsa 🛛 MM 1000.szs	Analysis Parameters>
	11 v run05Phil B02 062a 015.fsa MM 1000.szs	🕑 <analysis parameters=""></analysis>
	12 run05Phil D02 068a 016.fsa MM 1000.szs	Analysis Parameters>

19. Double click on a few sample files to visualize results, as shown on the example below. Dots in Dye/Sample Peak cells indicate peaks recognized as size standard peaks.

	Proi	[run05Phil_A ect Sample S		Windows Hel	n						
	:000	3000	-	000 6000	7000	8000	9000	10000	11000	12000	13000
		1					1	1			
600 _											
200											
300											
-											
400 _											
000											
300											
- 100											
200 _											
800								1			
								1			
400		A						1			
0						4.4	.	J	1.1.	. 1 k	1
BIG	YB	X: 13304 Y: 18	313 🔳								
Dye/Sample		Minutes	Size	Peak Height	Peak Area	Data Point	1				
Peak		Nanose P	0120	r cak r logn	T Call Pres	Data Form					
B, 1	-	77.67	586.14	2047	37868	9265					
G, 1	Λ	89.94	720.01	2809	57788	10729					
Y, 1	$I\Lambda$	83.59	648.37	1241	22086	9971					
R, 1	1.1	19.95	75.00	408	4717	2380					
R, 2	•	23.13	100.00	403	4225	2759					
R, 3		23.42	102.37	113	1341	2794					
R, 4	•	26.21	125.00	381	4589	3126					
R, 5	•	29.28	150.00	359	4510	3493					
		32.33	175.21	54	1199	3856					
R, 6					4365						
R, 7	•	35.29	200.00	310		4210					
R, 7 R, 8	٠	41.30	250.00	257	3248	4926	-				
R, 7 R, 8 R, 9	:	41.30 47.19	250.00 300.00	257 210	2884	4928 5629					
R, 7 R, 8 R, 9 R, 10	•	41.30 47.19 52.86	250.00 300.00 350.00	257 210 191	2884 3064	4926 5629 6305	** ** **				
R, 7 R, 8 R, 9 R, 10 R, 11	:	41.30 47.19 52.86 58.42	250.00 300.00 350.00 400.00	257 210 191 167	2884 3064 2362	4926 5629 6305 6968					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12	•	41.30 47.19 52.86 58.42 62.98	260.00 300.00 350.00 400.00 442.17	257 210 191 187 51	2884 3064 2362 656	4928 5629 6305 6968 7613					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 13	•	41.30 47.19 52.86 58.42 62.98 63.81	260.00 300.00 350.00 400.00 442.17 450.00	257 210 191 167 51 196	2884 3064 2362 650 3347	4928 5629 6305 6968 7513 7612					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 13 R, 14	•	41.30 47.19 52.86 58.42 62.98 63.81 66.40	250.00 300.00 350.00 400.00 442.17 450.00 475.00	257 210 191 167 51 196 189	2884 3064 2362 650 3347 4083	4926 5629 6305 6968 7613 7612 7920					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 13 R, 14 R, 15	•	41.30 47.19 52.86 58.42 62.98 63.81 66.40 69.02	250.00 300.00 350.00 400.00 442.17 450.00 475.00 500.00	257 210 191 167 51 196 189 184	2884 3064 2362 650 3347 4083 2840	4926 5629 6305 6968 7513 7612 7920 8233					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 14 R, 14 R, 16 R, 16	•	41.30 47.19 52.88 63.42 63.81 86.40 69.02 74.11	250.00 300.00 350.00 400.00 442.17 450.00 475.00 550.00	257 210 191 187 51 196 189 184 198	2884 3064 2362 650 3347 4083 2840 3093	4926 5629 6305 6968 7513 7612 7920 8233 8840					
R, 7 R, 8 R, 9 R, 11 R, 12 R, 13 R, 14 R, 14 R, 16 R, 16 R, 16	•	41.30 47,19 52,88 63,81 63,81 66,40 69,02 74,11 79,01	250.00 300.00 350.00 400.00 442.17 450.00 475.00 550.00 550.00 800.00	267 210 191 167 51 196 189 189 184 188 188 212	2884 3064 2362 656 3347 4083 2840 3093 4522	4926 5629 6305 6968 7513 7612 7920 8233 8840 9425					
R, 7 R, 8 R, 9 R, 11 R, 12 R, 13 R, 14 R, 16 R, 16 R, 17 R, 18	•	41.30 47.19 52.88 63.81 63.81 86.40 89.02 74.11 79.01 83.74	250.00 300.00 350.00 400.00 442.17 450.00 475.00 550.00 550.00 600.00 850.00	267 210 191 167 61 196 189 184 189 218 212 224	2884 3064 2362 656 3347 4083 2840 3093 4522 4227	4926 5629 6305 8968 7513 7612 7920 8233 8840 9425 9089					
R, 7 R, 8 R, 9 R, 11 R, 12 R, 14 R, 14 R, 16 R, 16 R, 16 R, 16 R, 16 R, 19	•	41.30 47.19 52.86 68.42 62.98 63.81 66.40 69.02 74.11 79.01 83.74 88.20	250.00 300.00 350.00 400.00 442.17 450.00 475.00 550.00 550.00 650.00 650.00 650.00 700.00	267 210 191 187 51 198 189 184 198 212 224 232	2884 3084 2362 656 3347 4083 2840 3093 4522 4227 4438	4926 5629 6305 6968 7613 7612 7920 8233 8840 9425 9969 10521					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 14 R, 14 R, 16 R, 16 R, 17 R, 18 R, 19 R, 20		41.30 47.19 52.86 58.42 63.81 86.40 69.02 74.11 79.01 83.74 88.20 92.49	250.00 300.00 350.00 400.00 442.17 450.00 475.00 500.00 550.00 650.00 650.00 650.00 700.00 750.00	267 210 191 187 51 196 189 184 198 212 224 232 220	2884 3064 2362 656 3347 4083 2840 3093 4522 4227 4438 4428	4920 5629 6305 6068 7513 7612 7920 8233 8840 9425 0989 10521 11033					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 13 R, 14 R, 16 R, 16 R, 16 R, 19 R, 20 R, 21	•	41.30 47.19 52.88 58.42 02.98 63.81 66.40 69.02 74.11 79.01 83.74 88.20 92.49 96.63	250.00 300.00 360.00 400.00 442.17 450.00 500.00 500.00 500.00 500.00 700.00 700.00 860.00 700.00 800.00	257 210 191 167 51 196 189 184 198 212 224 224 222 220 231	2894 3064 2362 666 3347 4083 2840 3093 4622 4227 4438 4428 4428 4428	4920 5629 6305 6068 7613 7612 7920 923 8233 8840 9425 9080 10521 11033 11515					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 12 R, 14 R, 14 R, 16 R, 16 R, 16 R, 19 R, 19 R, 20 R, 21 R, 22		4130 47,19 52,88 58,42 62,98 63,81 66,40 89,02 74,11 79,01 83,74 88,20 92,49 96,63 100,37	250.00 300.00 350.00 400.00 442.17 475.00 500.00 550.00 600.00 650.00 700.00 750.00 750.00 750.00 850.00	287 210 191 187 51 196 189 184 189 212 224 224 232 220 231 194	2884 3064 2362 056 3347 4063 3083 2840 3083 4522 4227 4438 4522 4227 4438 4224 3885	4920 5629 6305 6068 7613 7612 7920 8233 8840 9425 9099 10521 11033 11615 11073					
R, 7 R, 8 R, 9 R, 10 R, 11 R, 12 R, 13 R, 14 R, 16 R, 16 R, 16 R, 19 R, 20 R, 21		41.30 47.19 52.88 58.42 02.98 63.81 66.40 09.02 74.11 79.01 83.74 88.20 92.49 96.63	250.00 300.00 360.00 400.00 442.17 450.00 500.00 500.00 500.00 500.00 700.00 700.00 860.00 700.00 800.00	257 210 191 167 51 196 189 184 198 212 224 224 222 220 231	2894 3064 2362 666 3347 4083 2840 3093 4622 4227 4438 4428 4428 4428	4920 5629 6305 6068 7613 7612 7920 923 8233 8840 9425 9080 10521 11033 11515					

20. Click on the *l* button at the bottom of the window to control the size calling curve,

which should look as in the example shown below.



21. Close the window, then select File from the main menu, and Save as My project.

2.6 Editing sizing data and assigning alleles using Genotyper

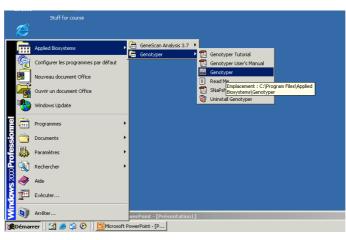
2.6.1 Principle

Size data obtained using GeneScan are edited and converted into MIRU-VNTR alleles, by using the Genotyper software. Therefore, customized files, called templates, are used for each multiplex (or for any of the 3 loci composing it). These templates contain allele-calling information for the corresponding loci, as well as macros that are used to automate the genotyping process.

2.6.2 Procedure

2.6.2.1 Opening templates and checking import preferences

1. Open the application from the desktop or the Windows menu by clicking

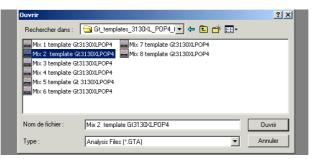




2. The Genotyper main menu is boxed below. Close the untitled file in the pop-up window.

Main	🖀 Genotyper 3.7	Click to
menu	File Edit Analysis Category Table Views Macro Window Help	close
	* Everything All peaks from scan 0 to 32000 in R/B/G/Y/O	
	Current Step Log	

 From the Genotyper main menu, select File and then Open. Select the Genotyper template corresponding to the multiplex (or individual loci) to be analyzed (see Table 2).

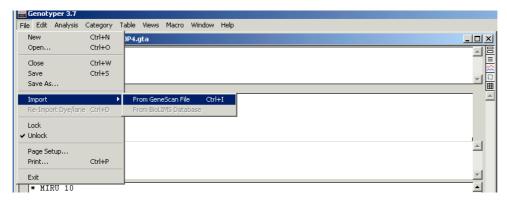


4. Before importation of GeneScan files, importation preferences <u>must be set as to</u> <u>import red color information</u> (corresponding to the size standard), in addition blue, green and yellow information for the PCR products. Therefore, from Genotyper main menu, select Edit and then Set Preferences. Make sure that Red is selected under Import colors. If not, tick the selection box as indicated, then click OK, then select Save from the main menu.

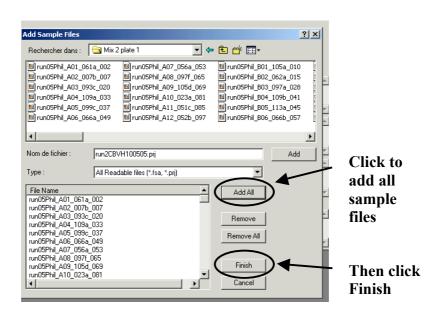
_	Set Preferences	<u>کا</u>
	* Options for exporting tables:	
	Field delimiter: 💿 Tab 🔿 Comma 🔿 Space 🔿 None	
	Line delimiter: 💿 CR 🔿 CR/LF 🔿 LF	
	* Additional windows to be opened with main window:	
	Dye/lane Plot Macro Statistics	
	🗖 Category 🧮 Table 🔲 Step	
	* Information to be shown in dye/lane list:	
	🔽 File name 🔽 Dye color 🔽 Sample Info	
	🔽 Lane number 🔲 Scale factor 🔲 Sample Comment	
T • 1 4	* Import colors:	
Tick to	🔽 🖉 Green 🔽 Yellow	
select	🔺 🔽 Red 🔲 Orange 📄 Import Raw Data	
	* Other options:	
	Double-clicking runs macros and steps	
	BioLIMS Cancel OK	

2.6.2.2 Importing GeneScan files

5. From the main menu, select File, then Import From GeneScan File.

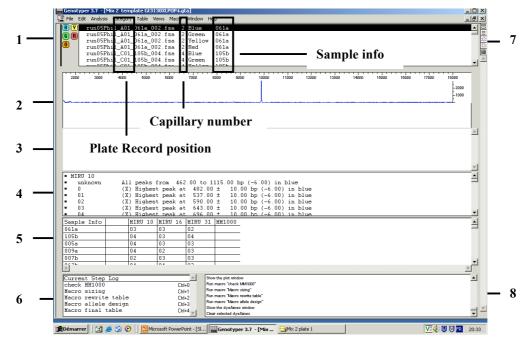


6. Select the folder containing GeneScan sample files corresponding to the multiplex to be analyzed. If the sample folder contains files analyzed for a single multiplex, then click Add All, and Finish. If the folder contains files from different multiplexes, select files corresponding to the Genotyper template by clicking+Ctrl the sample names, then click Add and Finish.



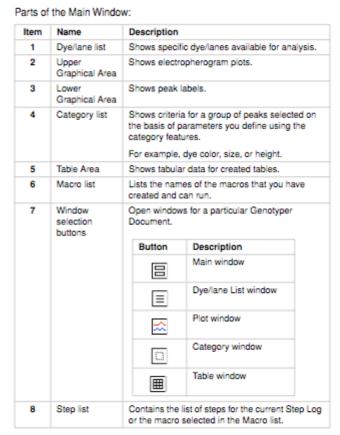
2.6.2.3 Main window and other windows of interest

7. The Genotyper Main Window should open as shown in the example below.



8. Parts of the Main Window are described below (from Genotyper User's Manual).

Parts of the Main The following table describes the part of the Main Window in the above Window figure.



9. The category Window, opened by clicking _____ contains the allele calling information specific for each multiplex. The example below corresponds to multiplex 1.

* MIRU 04	4
* unknow	
* 0	(X) Highest peak at 175.00 ± 10.00 bp (-5.00) in blue
* 0'	(X) Highest peak at 122.00 ± 10.00 bp (-3.00) in blue
* 1	(X) Highest peak at 252.00 ± 10.00 bp (-7.00) in blue
* 1'	(X) Highest peak at 199.00 ± 10.00 bp (-6.00) in blue
* 10	(X) Highest peak at 945.00 ± 10.00 bp (-27.00) in blue
* 11	(X) Highest peak at 1022.00 ± 10.00 bp (-29.00) in blue
* 12	(X) Highest peak at 1099.00 ± 10.00 bp (-31.00) in blue
* 13	(X) Highest peak at 1176.00 ± 10.00 bp (-33.00) in blue
* 14	(X) Highest peak at 1253.00 ± 10.00 bp (-35.00) in blue
* 15	(X) Highest peak at 1330.00 ± 10.00 bp (-37.00) in blue
* 2	(X) Highest peak at 329.00 ± 10.00 bp (-11.00) in blue
* 2'	(X) Highest peak at 276.00 ± 10.00 bp (-7.00) in blue
* 3	(X) Highest peak at 406.00 ± 10.00 bp (-14.00) in blue
* 3'	(X) Highest peak at 353.00 ± 10.00 bp (-14.00) in blue
* 4	(X) Highest peak at 483.00 ± 10.00 bp (-12.00) in blue
* 5	(X) Highest peak at 560.00 ± 10.00 bp (-15.00) in blue
* 6	
0	
,	(X) Highest peak at 714.00 ± 10.00 bp (-21.00) in blue (X) Highest peak at 701.00 ± 10.00 bg (-22.00) in blue
0	(X) Highest peak at 791.00 ± 10.00 bp (-23.00) in blue
,	(X) Highest peak at 868.00 ± 10.00 bp (-25.00) in blue
* MIRU 26	
unknow	
* 00	(X) Highest peak at 285.00 ± 10.00 bp (-5.00) in green
* 01	(X) Highest peak at 336.00 ± 10.00 bp (-5.00) in green
* 02	(X) Highest peak at 387.00 ± 10.00 bp (-5.00) in green
* 03	(X) Highest peak at 438.00 ± 10.00 bp (-5.00) in green
* 04	(X) Highest peak at 489.00 ± 10.00 bp (-5.00) in green
* 05	(X) Highest peak at 540.00 ± 10.00 bp (-5.00) in green
* 06	(X) Highest peak at 591.00 ± 10.00 bp (-5.00) in green
* 07	(X) Highest peak at 642.00 ± 10.00 bp (-5.00) in green
* 08	(X) Highest peak at 693.00 ± 10.00 bp (-5.00) in green
* 09	(X) Highest peak at 744.00 ± 10.00 bp (-5.00) in green
* 10	(X) Highest peak at 795.00 ± 10.00 bp (-5.00) in green
* 11	(X) Highest peak at 846.00 ± 10.00 bp (-5.00) in green
* 12	(X) Highest peak at 897.00 ± 10.00 bp (-5.00) in green
* 13	(X) Highest peak at 948.00 ± 10.00 bp (-5.00) in green
* 14	(X) Highest peak at 999.00 ± 10.00 bp (-5.00) in green
* 15	(X) Highest peak at 1050.00 ± 10.00 bp (-5.00) in green
* MIRU 40	
* unknow	All peaks from 334.00 to 1115.00 bp (-1.00) in yellow
* 0	(X) Highest peak at 354.00 ± 10.00 bp (-4.00) in yellow
* 1	(X) Highest peak at 408.00 ± 10.00 bp (-8.00) in yellow
* 10	(X) Highest peak at 894.00 ± 10.00 bp (-49.00) in yellow
* 11	(X) Highest peak at 948.00 ± 10.00 bp (-54.00) in yellow
* 12	(X) Highest peak at 1002.00 ± 10.00 bp (-59.00) in yellow
* 13	(X) Highest peak at 1056.00 ± 10.00 bp (-64.00) in yellow
* 14	(X) Highest peak at 110.00 ± 10.00 bp (-69.00) in yellow
* 15	(X) Highest peak at 1164.00 \pm 10.00 bp (-74.00) in yellow
* 2	(X) Highest peak at 462.00 ± 10.00 bp (74.00) in yellow (X) Highest peak at 462.00 ± 10.00 bp (-12.00) in yellow
* 3	(X) Highest peak at 516.00 ± 10.00 bp (-12.00) in yellow
* 4	(X) Highest peak at 570.00 ± 10.00 bp (-10.00) in yellow (X) Highest peak at 570.00 ± 10.00 bp (-20.00) in yellow
* 4 * 5	(X) Highest peak at 570.00 ± 10.00 bp (-20.00) in yellow (X) Highest peak at 624.00 ± 10.00 bp (-25.00) in yellow
* 6	(X) Highest peak at 624.00 ± 10.00 bp (-25.00) in yellow (X) Highest peak at 678.00 ± 10.00 bp (-30.00) in yellow
* 6 * 7	
	(X) Highest peak at 732.00 ± 10.00 bp (-35.00) in yellow (X) Highest peak at 786.00 ± 10.00 bp (-20.00) in yellow
0	(X) Highest peak at 786.00 ± 10.00 bp (-39.00) in yellow (X) Highest peak at 840.00 ± 10.00 bp (-44.00) in sellow
* 9	(X) Highest peak at 840.00 ± 10.00 bp (-44.00) in yellow

Note: \pm x bp and (- y bp) define size tolerance and size offsets (see 2.7) for allele identification, respectively; blue, green and yellow define the dye labeling for the respective loci. The Unknown category finds and labels any peaks that do not belong to any of the allelic bins, but are within the size range for that locus. Unknown labels are useful hints for detection of potential problematic peaks.

10. The Category Window also contains the information for size standard peak labeling,

common to all multiplexes.

* MM1000			
* 025	(X) Highest peak at 2	$5.00 \pm$	0.50 bp in red
* 050	(X) Highest peak at 5		0.50 bp in red
* 075	(X) Highest peak at 7		0.50 bp in red
* 100	(X) Highest peak at 10		0.50 bp in red
* 1000	(X) Highest peak at 10		0.50 bp in red
* 125	(X) Highest peak at 12		0.50 bp in red
* 150	(X) Highest peak at 15	$50.00 \pm$	0.50 bp in red
* 200	(X) Highest peak at 20	$\pm 00.00 \pm$	0.50 bp in red
* 250	(X) Highest peak at 25	$50.00 \pm$	0.50 bp in red
* 300	(X) Highest peak at 30		0.50 bp in red
* 350	(X) Highest peak at 35	$50.00 \pm$	0.50 bp in red
* 400	(X) Highest peak at 40	$\pm 00.00 \pm$	0.50 bp in red
* 450	(X) Highest peak at 45	$50.00 \pm$	0.50 bp in red
* 475	(X) Highest peak at 47	$75.00 \pm$	0.50 bp in red
* 500	(X) Highest peak at 50	$\pm 00.00 \pm$	0.50 bp in red
* 550	(X) Highest peak at 55	$50.00 \pm$	0.50 bp in red
* 600	(X) Highest peak at 60	$\pm 00.00 \pm$	0.50 bp in red
* 650	(X) Highest peak at 65	$50.00 \pm$	0.50 bp in red
* 700	(X) Highest peak at 70	$\pm 00.00 \pm$	0.50 bp in red
* 750	(X) Highest peak at 75	$50.00 \pm$	0.50 bp in red
* 800	(X) Highest peak at 80	$\pm 00.00 \pm$	0.50 bp in red
* 850	(X) Highest peak at 85	$50.00 \pm$	0.50 bp in red
* 900	(X) Highest peak at 90	$\pm 00.00 \pm$	0.50 bp in red
* 950	(X) Highest peak at 95	$50.00 \pm$	0.50 bp in red

11. The Macro Window contains information about shortcut commands (Ctrl+0, etc) to run macros, and corresponding operation steps. These operations can be viewed by clicking macros in this Window, and are summarized in the Table below.

Macro	Command	Action
Check MM 1000	Ctrl+0	Labels and visualizes size standard peaks with size in bp
Sizing	Ctrl+1	Labels, filters and visualizes locus peaks with size in bp (see below), and writes intermediate result table
Rewrite table	Ctrl+2	Updates intermediate result table after data editing
Allele design	Ctrl+3	Converts sizes into alleles
Final table	Ctrl+4	Writes a final table

12. Filtering steps in the sizing macro (operated by pressing Ctrl+1) are described below. The "Remove labels from peaks in the size range xx to yy bp" step is the only macro step that differs between multiplex templates.

Steps	Effect
Remove labels from peaks in the size range 0.00 to 90.00	Removes labels in size range below size of the smallest
bp	amplicon in any locus in multiplex
then remove labels from peaks whose height is less than	Removes labels from <u>SUSPECTED</u> noise and stutter
32% of the highest peak in a category's range	peaks
then remove labels from peaks preceeded by a higher,	Removes labels from <u>SUSPECTED</u> shoulder peaks, if
labeled peak within 0.00 to 10.00 bp	peak height is lower than allele peak
then remove labels from peaks followed by a higher,	Removes labels from <u>SUSPECTED</u> shoulder peaks, if
labeled peak within 0.00 to 10.00 bp	peak height is lower than allele peak

Note: when PCR signals are strong and cause frequent pull-up peaks, peak filtering level can

be set to 60 % instead of 32 %, as follows:

- a) Select Macro sizing from the main window, and from menu, select Macro>Edit Step.
- b) Replace 32 % by 60 % in the % of the highest peak in a category's name box, and click
 - Replace.

Image: Search and add add add add add add add add add	Genotyper 3.7 - [Mix A Run51.gta] File Edit Analysis Category Table Views Macro Window H Tun03Mara_H01_443_013.fs Vecord Steps run03Mara_H01_443_013.fs Vecord Steps run03Mara_H01_443_013.fs Vecord Steps run03Mara_H01_443_013.fs Vecord Steps run03Mara_H02_302_025.fs Run Macro run03Mara_H02_302_025.fs Change Macro N run03Mara_H02_302_025.fs Change M	ame	Edit step then change %
Image: Constraint of the set of the	2000 2000 3000 4000 4000 4000 Edit Step Run Step Add Comment Clear Step Log		Remove labels from peaks in the size range
* MIRU 40 MIRU 40 MIRU 40 MIRU 00 MIRU 40 MM1000 MIRU 40 Overflow MIRU 66 2 05 1 200 1 1 1 64 2 05 1 200 1 1 12 2 Not Found 1 200 1 109 2 05 1 200 1 611 3 04 1 200 1 11a 2 0 1 200 1 Current Step Log check M1000 Cit/b0 Cierra Labels Cierra Labels	 10 (X) Highest peak at 795 0 11 (X) Highest peak at 846 0 12 (X) Highest peak at 897 0 13 (X) Highest peak at 948 0 14 (X) Highest peak at 949 0 	0 ± 10.00 bp (-5.00) in gr 0 ± 10.00 bp (-5.00) in gr	highest peak in a category's range Image: The move labels from peaks preceeded by higher, labeled peak within 0.00 to 3.00 bp □ (Higher by at least 5 %) Image: The move labels from peaks followed by higher, labeled peak within 0.00 to 3.00 bp □ (Higher by at least 5 %)
Macro Mix A Sizing Ctt+1 Select all color lanes	★ MIRU 40	M1000 MIRU 04 Overflow MIRU 00	

2.6.2.4 View, dye/lane sorting, and peak labeling options

- 13. From Genotyper main menu, select Views, then Display by Scan. Note: <u>Important.</u> Display problems may appear if results are displayed by sizes, due to possible inconsistencies among sizing data obtained from some capillaries.
- 14. Select Views from Genotyper main menu, then Dye/Lane Sorting, then select display Sample name, Dye color, and Sample Info as criterions 1, 2, and 3, respectively, from the pull-down menus shown below, and press OK. *Note:* for 3700 or 3730 systems, sorting by Lane number/Dye color/Sample Info is required for detection of possible "cross-talk" effects (see below) between adjacent capillaries.

	7000	8000	9000	10000	11000	12000	, 0	13000	14000
	🚟 Dye/La	ne Sortin	g						×
	Sort dye/la	ine list in fol	lowing order:						
	Precedenc	e Ite	em		Sorto	order			
	1.		Lane numb	er 🔻	• A	scending	0	Descend	ling
	2.		Dye color	•	ΘA	scending	0	Descend	ling
	3.		Sample Info	•	• A	scending	0	Descend	ling
	4.			•	• A	scending	0	Descend	ling
	5.			•	• A	scending	0	Descend	ling
Ì						Cancel		ÖK	

2.6.2.4a. Entering Sample Info (only when using Data Collection version 3.x)

Entering sample info is mandatory for construction final result table in Genotyper. Unfortunately, it is not possible to enter this info in the plate record (see page 25), when using Data Collection software version 3.x. To by-pass this problem, this info must be entered for each sample file at the level of Genotyper. Therefore,

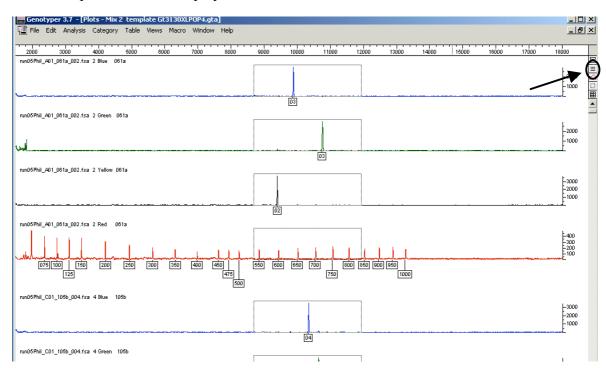
15. Open the Dye/Lane List window.

- 16. In the list, select successively <u>each Lane for each Dye</u> and enter the corresponding sample name into Sample Info box in the top of the Dye/Lane window, as shown below. <u>Beware of clerical errors !</u>
- 17. When finished, select Save As from main Menu>File, and save as your experiment multiplex x in the appropriate folder.

Genotyper 3.7 - [Dye/lanes - Mix A F			Wedew	-	_ 미 › _ 리 ›
File Edit Analysis Category Table Sample Info:	views	Macro	WIIIUUW	neip	- 0 -
66					
Sample Comment					
		51			
run03Mara_A01_66_002.isa		Blue	66		
run03Mara_A01_66_002.fsa		Green	66		
run03Mara_A01_66_002.fsa		Yellow Red			
run03Mara_A01_66_002.fsa	_		64	Enter sample	
run03Mara_A02_64_006.fsa		Blue Green		-	
run03Mara_A02_64_006.fsa	-		64	name into Sample	
run03Mara_A02_64_006.fsa		Yellow Red	64 64	Info box for each	
run03Mara_A02_64_006.fsa		Rea Blue	12		
run03Mara_A03_12_018.fsa		Green	12	dye and for each	
run03Mara_A03_12_018.fsa		Yellow		sample	
run03Mara_A03_12_018.fsa		Red	12 12	sample	
run03Mara_A03_12_018.fsa		Rea Blue	109		
run03Mara_A04_109_033.fsa		Green	109		_
run03Mara_A04_109_033.fsa					
run03Mara_A04_109_033.fsa		Yellow Red			
run03Mara_A04_109_033.fsa			$109 \\ 611$		
run03Mara_A05_611_037.fsa		Blue Green			
	37	Green	611		

2.6.2.5 Running Macros and viewing results

- 18. Press Ctrl+0, immediately followed by Ctrl+1, Ctrl+2, and Ctrl+3. Wait a while until the process is finished. *Note:* These Macros can be run and their action viewed separately.
- 19. An example of result is displayed below.

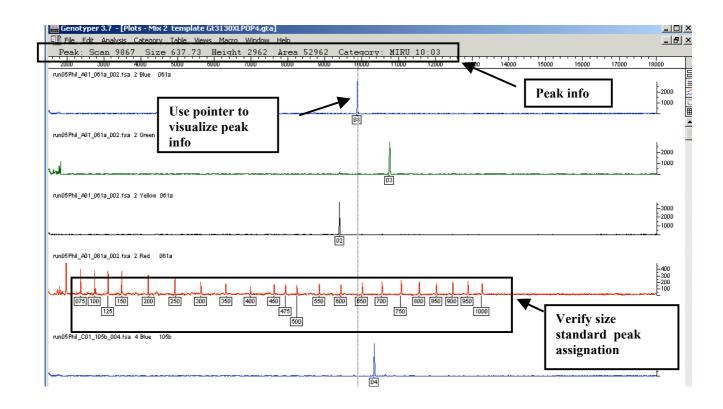


20. When using a PC Genotyper version, a bug in plot display appears when analyzing many sample files in a single template. To overcome it, dye/lanes must be analyzed by halves. Therefore, click the Dye/Lane Window button, then select one first half of the dye lanes as shown below, by clicking the first Dye/Lane and then Shift+clicking the last Dye/Lane. Notice the last sample of this half.

Cond	tunar	3.	7 - IDs	/an	м. – М	iu 2 I	anan	ata Ct21	30XLPOP	d obal
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ampier	nio.									
ample (Commer	nt								
		_								
7:110	15Pb		HO4	155c	044	fea	4.4	Red	055c	
								Blue	113a	
				113a				Green	113a	
run	05Phi	n	B05	113a	045	fsa	45	Yellow	113a	
\mathbf{run}	05Phi	11	BOS	113a	045	.fsa	45	Red	113a	
run	05Phi	11_	D05_	113c	046	.fsa	46	Blue	113c	
								Green		
								Yellov		
				113c					113c	
				113e					113e	
								Green		
									113e	
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				113g				Blue		
				113g				Green		
				113g 113g				Yellov	r 113g 113g	
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				066a				Green		
				066a				Vellov		
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	0.01.11.2		C04	100-	0.00	4	E0.	v_11		

2.6.2.6 Verifying size standard assignation and peak information

- 21. For each lane/capillary, verify that the size standard assignation is correct, *i.e.* all peaks within the analysis range (defined by data points in GeneScan analysis, see 2.5.2.2) must be labeled correctly as indicated in the example below.
- 22. Verify the presence of allele label for each colour. Unless some clonal variant subpopulation or mixed DNA population are present (see below), <u>one label is expected per color. If pull-up peaks (see 2.6.2.7.2) are too frequent, change the peak filtering level in the sizing macro (see 2.6.2.7.2) and rerun macros as above.</u>
- 23. Peak information, including the positions in scan numbers, the size in bp, the peak height and area, and the corresponding allele (category), can be visualized for each color detected a capillary, by placing the cross-hair cursor on the peak of interest as shown below.



2.6.2.7 Detecting and removing Genotyper labels from problematic peaks

Occasionally, size standard assignation may be incorrect. Consequently, sizes/alleles are not assigned correctly for PCR products.

In addition, despite filtering by using the sizing macro, various kinds of artifactual peaks may be labeled. Such peaks include:

- "Pull-up" peaks, resulting from spectral overlap from another dye. Such peaks may result from the presence of large amounts of a particular dye-labeled PCR product, or from sub-optimal spectral calibration, or from both causes.
- Peak shoulders or "mirror" peaks in a same dye. Such peaks result from the presence of large amounts of a particular dye-labeled PCR product, and are therefore often associated with pull-up peaks in other dyes.
- Spurious peaks. Such peaks are often thin peaks, which can be observed at a same position across several dyes.
- 4) Peaks resulting from fluorescence cross-talk between flanking capillaries (on <u>3700</u> or <u>3730 sequencers</u>). Such peaks result from the presence of very large amounts of PCR products in a proximal capillary. They should not observed on 4-, or 16capillaries 3100/3130 sequencers.
- 5) Stutter peaks, classically caused by slippage of the polymerase enzyme during PCR.

Importantly, peaks of types 1) to 4) are frequently tagged with "unknown" labels, as their size do not belong to any of the allelic bins. **Thus, the presence of "unknown" labels particularly**

prompts for attention.

<u>However, this rule is not systematic, as some false peaks can fall into an allelic bin by</u> <u>chance, and can therefore be tagged with allelic labels.</u>

Labels from all types mentioned above should be removed, according to algorithms explained below.

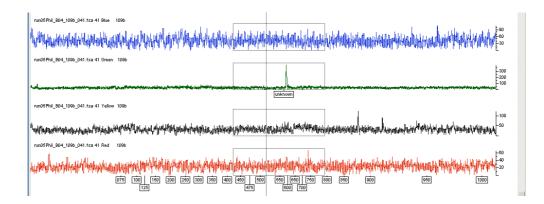
The procedure for label removal is the following:

- 1. Place the cross-hair cursor on the peak of interest. The vertical peak locator will be aligned with the midpoint of the peak.
- 2. Click <u>once</u> to remove the label from any incorrectly sized, artifactual, or stutter peak.

2.6.2.7.1 Incorrectly sized peaks

If size standard assignation is incorrect for results from a capillary, i.e. if peaks within the analysis range (defined by data points/scan numbers defined in GeneScan analysis parameters, see 2.5.2.2) are not labeled correctly, sizes/alleles will not be assigned correctly for the corresponding PCR products. Such a problem may for instance happen consecutively to a sample injection problem, resulting in low or absence of size standard peaks and incorrect size standard assignation to background signals, as shown in the example below (in this case the peak height detection threshold was set to 50).

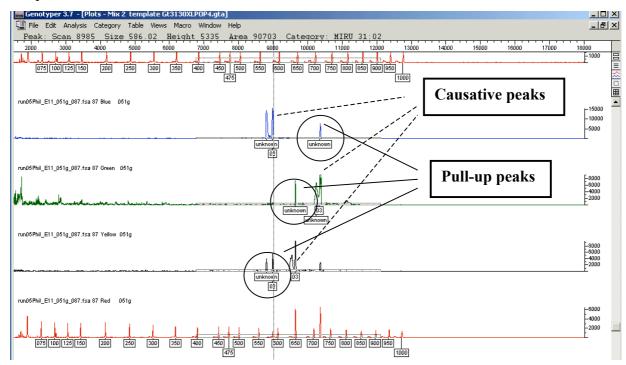
- 1. Inspect the size standard label pattern.
- In case of size assignation problems, remove the labels, corresponding to either unknown or apparently recognized alleles, from all PCR product peaks from the same capillary.



2.6.2.7.2 Pull-up peaks

"Pull-up" peaks result from spectral overlap from another dye within a same capillary. They can be diagnosed, as they usually appear as peaks exactly co-migrating with a strong peak labeled with another dye.

Diagnostics is especially facilitated when 1) pull-up peaks are labeled as "unknown", <u>and 2</u>) another peak tagged with the same dye is labeled as a recognized allele, <u>and 3</u>) the causative peak labeled with a different dye displays itself shoulder or mirror peaks indicative of signal saturation, as in the example shown below.



- Systematical observation of pull-up peaks caused by strong genuine PCR products, is indicative of excess of PCR products. In this case, reduce genomic DNA concentration, PCR cycle numbers, or dilute PCR products before addition to the loading mix.
- Systematical observation of pull-up peaks, even when the heights of causative true peaks are small, is indicative of sub-optimal spectral calibration. Reperform spectral calibration.
- 3. More generally, labels from co-migrating, potential pull-up peaks can be detected and interpreted according to the algorithm shown in Figure 16.

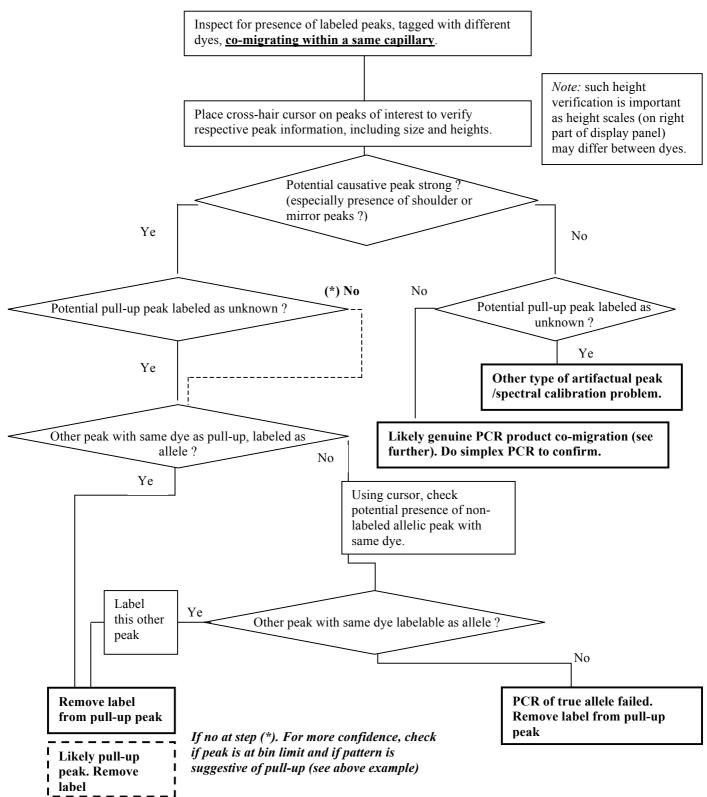
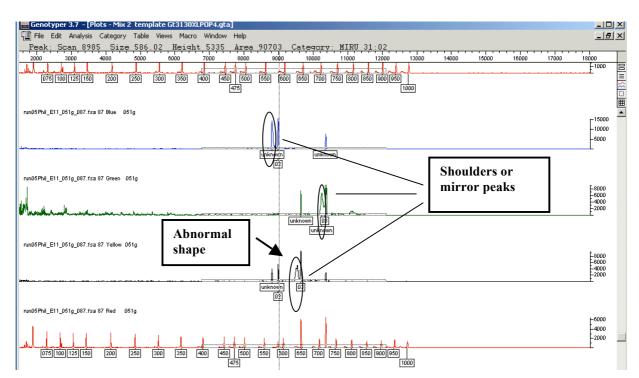


Figure 16 Algorithm for interpretation of potential pull-up peaks

2.6.2.7.3 Peak shoulders or mirror peaks

The presence of large amounts of a particular dye-labeled PCR product may cause the appearance of shoulders besides the genuine allelic peak. Furthermore, so-called mirror effects may occur, resulting in apparent and typical digging of the central part of large peaks, and even apparent reduction of the peak boundaries (see example below).

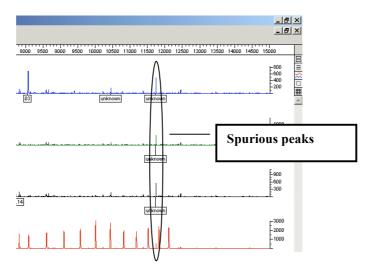
In most cases, the boundary with the lowest apparent size in bp is labeled as unknown, while the boundary with the largest size in bp is recognized as a true allele. <u>However, in extreme cases,</u> <u>the two boundaries may be separated by apparent sizes as large as one repeat length, and may</u> <u>thus appear as two independent allelic peaks at first glance.</u> Therefore, the diagnosis of such peaks is also based on their shape, and with frequent association with the presence of pull-up peaks in other dyes.



- 1. Remove labels from obvious peak shoulder or mirror peaks, initially assigned as unknown.
- 2. Check the presence of mirror peaks with abnormal shapes as seen above, initially assigned as alleles. Remove their labels.



Spurious peaks appear as thin peaks, that may be observed at a same position across several



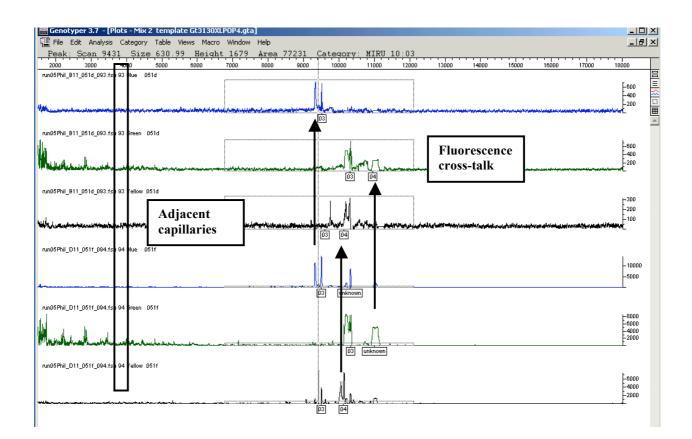
dyes within a capillary. It may be caused by the presence of a bubble during migration.

- 1. Check for the presence of any labeled thin peaks, observed at identical positions across all dyes.
- 2. Remove their labels.

2.6.2.7.5 Cross-talks between flanking capillaries (for 3700 and 3730)

Artifactual peaks may occur in a capillary, when very large amounts of PCR products are present in the adjacent capillary (see example below). This phenomenon is described as fluorescence cross-talk. It is similar to "contamination" effects, which may be seen between adjacent gel lanes when using a 377 sequencer. <u>Cross-talk effects may be observed on 96-capillaries 3700 or 3730 sequencers, but should not observed on 4-, or 16-capillaries 3100 sequencers.</u>

- 1. Compare patterns from immediately adjacent lanes/capillaries
- 2. Check for the possible occurrence of exactly co-migrating peak patterns, between a lane containing labeled weak peaks and the immediately proximal lane (see below).
- 3. Remove the corresponding labels, if any.



2.6.2.7.6 Stutter peaks

As indicated in 2.3.3, stutter peaks can be easily diagnosed, as they appear most often as a regular ladder of much lower intensity peaks (see example A below), corresponding to sizes of PCR fragments that lack one or more repeats, or more rarely that contain one or more additional repeat. Therefore, as for electrophoretic analysis using agarose gels, the positions of stutter peaks can be used to confirm the allelic assignation of the principle fragment (see point 5 in 2.3.2).

However, sub-optimal amplification of some loci, especially with larger repeat numbers, may sometimes result in stronger intensities of stutter peaks (example B). Exceptionally, band ladder with no clearly sharpest band can occur, preventing any allelic assignation (example C).

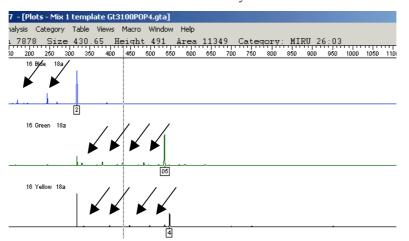
Based on analysis of appropriate controls, we have determined a rule for correctly distinguishing true alleles from stutter peaks as follows:

- The true allele is assigned as the peak having the highest repeat number, <u>bevond</u> which a sharp decrease is observed in heights of further stutter peaks (if any). If further stutter peaks are present, they must be uniformly small.
- 2. Remove labels from other peaks, if any.

3. If a "bell-shaped" distribution of band intensities is observed without any clear sharp

band at the extremity of the ladder, remove all labels and re-amplify.

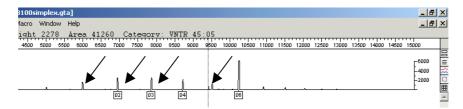
Example A: usual stutter pattern



Stutters are indicated by arrows

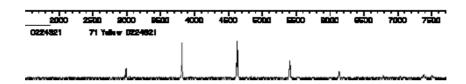
Example B: more complex stutter pattern

Stutters are indicated by arrows. The corresponding locus (VNTR 1982) is not included in our panel.



Example C: non-interpretable stutter pattern

The corresponding locus (VNTR 1982) is not included in our panel.



2.6.2.8 Adding labels to true alleles

Due to the label filtering process, true alleles may occasionally not be labeled. This can typically occur when some intense pull-up peaks are observed. In this case, because of the sizing macro parameters, true alleles with a peak height less than 32% of these pull-up peaks will not be labeled. The corresponding peaks must then be labeled manually.

<u>In such conditions, because of disproportionate impact of pull-up peaks on the plot</u> <u>scale, these true alleles might remain unnoticed, especially when they correspond to large</u> <u>repeat numbers. Therefore, particular attention must be paid to detect them.</u>

- 1. When intense pull-up or other artifactual peaks are present, inspect the plot patterns according to the algorithm in Figure 16, by moving the cursor along the whole analysis range to facilitate detection of potential true alleles.
- 2. Taking into account Place the cross-hair cursor on the peak of interest. The vertical peak locator will be aligned with the midpoint of the peak.
- 3. Click <u>once</u> to add a label to the true peak.
- 4. Press Ctrl+3 to change the size label into an allele label.

2.6.2.9 Genuine co-migration between PCR products tagged with different dyes

Depending on the polymer used, nearly exact co-migration of two genuine PCR products labeled with different dyes can occur within some multiplex (*e.g.* allele 2 for loci 47 and 52 in our conditions). This can be predicted, after size calibration and application of appropriate size offsets, from comparison of the allele-calling table between the respective loci within the different multiplexes. However, it is sometimes to distinguish genuine co-migration from pull-up effects

- To distinguish between these two possibilities, use the algorithm in Figure 16.
 Allelic labels can be assigned only if both respective peaks have moderate heights.
- 2. In case of doubts, re-amplify the two loci by simplex PCR, and analyze them separately.

2.6.2.10 Genuine double alleles

After application of the filtering process described above, genuine double alleles can be observed in certain cases. If 2 alleles are concordantly observed in several independent MIRU-VNTR loci for a given sample, this indicates the presence of a mixed DNA population in this sample. This mixed population can result from a true mixed infection, or from culture or DNA contamination. In contrast, the occurrence of 2 alleles is observed in a single locus rather suggests the presence of a given allelic variant within a clonal isolate.

2.6.2.11 Viewing and analyzing the second half of the capillary lanes

If applicable, the second half of the capillary lanes can be analyzed according to the same procedure as described above.

To select this half, click the **Dye/Lane Window** button, then select the appropriate dyes, by clicking the dyes from the first lane and then **Shift+clicking** the dyes from the last lane. *Note:* when using a 96-capillary system, include also the last sample from the first half, to control the occurrence of potential fluorescence cross-talks.

2.6.2.12 Final sorting by file names

After analysis of all capillaries, all sample files should be sorted by file names, as indicated below. This mode of final sorting choice is more reliable and convenient to compare result tables from different analysis runs, among which sample plate mappings are not necessarily identical.

- To select all samples, click the Dye/Lane Window button, then press Ctrl+A to select all lanes.
- 2. From Genotyper main menu, select Views, then Dye/Lane Sorting, then select Sample Name and Dye Color from the first and second pull-down menus, respectively, both in ascending order, and then press OK. *Note:* this sorting can also be performed <u>after</u> the creation of the final results table, by selecting Table from the main menu, then Sort Table, and selecting Sample Info from the first pull-down menu.

2.6.2.13 Creating final results tables and verifying results

Final results tables (see example below) are created using a macro called "final table" in the Genotyper templates. These tables are used to check, and subsequently export the results.

Columns of interest in this final table include the following information:

- Sample information

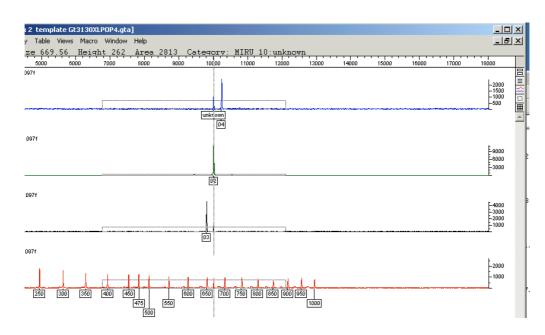
- Peak label information for the 3 loci for each sample. The expected information corresponds to alleles. In contrast, <u>"Unknown" tags are unexpected and prompt for attention</u>. "Not Found" tags are displayed when no label/result could not be assigned for some loci.
- "Overflow" status for each locus. "Check" tags (see below) indicate the presence of two or more alleles in a given locus, which is normally unexpected and prompts for attention.

🛄 File Edit An	alysis Category	Table Views	Macro Window	Help									
Sample Info	MIRU 10	MIRU 16	MIRU 31	MM1000	MIRU	10	Overflow	MIRU	16	Overflow	MIRU	31	Overflow
061a	03	03	02	075									
007Ъ	02	03	03	075									
093c	03	02	03	075									
109a	04	02	03	075									
099c	03	04	03	075									
066a	05	03	03	075									
056a	04	03	03	075		_							
097f 🕻	unknown	02	03	075 🤇	check)						
105d	09	03	03	075			-						
023a	05	03	03	075									
051c	05	03	03	075									
052Ъ	03	02	03	075									
105a	03	02	03	075									
062a	05	03	03	075									
097a	03	04	03	075									
109Ъ	Not Found	Not Found	Not Found	075									
113a	03	03	04	075									

To create a final table and verify the results, proceed as follows:

- 1. Make sure that all lanes are selected in the Dye/Lane Windows.
- 2. Press Ctrl+4.
- 3. Click the Table window button.
- 4. If any "unknown" or "check" tags are present in locus or locus overflow columns, respectively, click the corresponding "sample info" cell to visualize the peak patterns.
- 5. If appropriate, remove labels as described in 2.6.2.7.
- 6. Update the table by pressing **Ctrl+4**.
- When all results are checked, select File from the main menu, then Save as Gt_Results_mixz_runx in an appropriate folder.

The following image shows verification of the sample with "unknown" and "check" labels in the example above. In this case, the presence of these tags were indicative of a pull-up peak with the blue dye, clearly caused by a strong peak with the green dye.



2.6.2.14 Exporting final table to Excel

- 1. From the main menu, select **Table**, then **Export to File**, and **Save** as Results mixz runx.
- 2. From Excel main menu, select Open, then All Types of Files.
- 3. Import the table, by clicking twice Next, and then Finish.

2.7 Size offset calibration

Differences in relative migration can occur between the size standard and the amplicons on the polymer depending on the polymer used for electrophoresis, and on the locus. In some loci, these differences can be fixed, or while in others it increases linearly upon the number of repeats. Also depending on the locus, offsets may be negative or positive. Therefore, <u>it is essential to take this effect into account, otherwise allelic assignation in terms of actual repeat numbers will be wrong</u>.

Application of size offsets in the Genotyper allele calling information correct these differences. These offsets are determined experimentally, by using reference PCR products with

alleles and sizes pre-determined using genomic data (*e.g.* from H37Rv), sequence analysis, or by using agarose gel.

Using identical electrophoretic conditions (*i.e.* essentially the same polymer and run temperature), we have observed that differences in size offsets are minimal for most loci between different sequencers of a same or comparable type (*i.e.* between 3100s, or between a 3100 and a 3130 XL). However, more important differences are seen with a few loci. <u>Therefore, size offsets</u>

must be verified experimentally.

- Select reference PCR products to cover the allelic range in the different loci, in order to determine if offsets increase upon repeat numbers. Alternatively, using stutter peaks from large alleles can to reduce the number of analyses.
- 2. Using the allele calling information in the Genotyper templates, compare the sizes observed by capillary electrophoresis with those predicted from sequence analysis, or from alleles carefully pre-determined by electrophoresis using agarose gels.
- 3. In Genotyper, click the Category Window button.
- 4. If the size offset is fixed upon repeat numbers, select all alleles/categories in the locus of interest, then select **Category** from the main menu, and **Offset categories**.
- 5. If the size offsets differ upon repeat numbers, select allele/categories separately.
- 6. Introduce the corresponding offsets.
- When all offsets have been introduced, save the new template with a different name.
 These new templates can now be used for MIRU-VNTR analysis on your sequencer.

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4 Appendixes

154 MIRU 02 424 VNTR 42 577 VNTR 43 ETRC 580 MIRU 04 ETRD 802 MIRU 40 960 MIRU 10 1644 MIRU 16 2059 MIRU 20 2163b QUB-11b 2165 ETRA
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2165 ETRA
2347 VNTR 46
2401 VNTR 47
2461 VNTR 48 ETRB
2531 MIRU 23
2687 MIRU 24
2996 MIRU 26
3007 MIRU 27 QUB-5
3171 VNTR 49
3192 MIRU 31 ETRE
3690 VNTR 52
4052 QUB-26
4156 VNTR 53 QUB-41566
4348 MIRU 39

4.1 Conventional and alias designations of MIRU-VNTR loci

^aMIRU-VNTR loci are listed according to their position in kbp on the H37Rv genome

Multiple Locus Alias Repeat unit length, bp PCR primer pairs (5' to 3', with labeling indicated') Wix 1 580 MRU 4 77 GCCCAGAGAGCCCCAACTCG (FAM) GCCCAGCAGACAGCCCCAACTCG (FAM) 2996 MIRU 26 51 CATCAGCCCCAACAGGCCAACAGGCCAACAGGCCAACAGGCCAACAGGCCAACAGGCCAACAGGCCAACAGGCAATAG 802 MIRU 40 54 GGGGTGATCTGCGGAAATCGCAACAGGTG (FNDD) GGGTGATCGGGCCACACGCGCAGCAGCCAGCAGCCAGGCAATAG Mix 2 960 MIRU 16 53 CCCGTGGTGACCAACGGCATTCACACAGTGC (FCAACTTGGACTCACTCACTACCAGTAA Mix 3 1644 MIRU 16 53 CCGGTGAGCCAGCAGGCAGTTCATACGGACTTAA CCCGTGGGCAGTGGCTCTAACGCAGTAATTTTAA GGCCACACGCGGGATCATCAGCCCAGTACTTAAT Mix 3 424 42 51 CGAAGCGCGGGAAATCGTCATC (FAM) Mix 4 2010 47 58 CGAAGGCGGGGCAAGGCGGGGAAATCGTCATC (FAM) Mix 4 2011 47 58 CGGTGGACCCCCCCCTTCTTCATC (FAM) Mix 5 2165b FTR A 75 AAATGGCCCCGAACTCTCTCTCT (FAM) Mix 6 2014 77 58 CGGTGGAGCCCCACTCTCTCTCT (FAM) Mix 7 2063b QUB-11b 69 <td< th=""><th></th><th>-</th><th>ner sequences</th><th></th><th></th></td<>		-	ner sequences		
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InterpretationInterpretationInterpretation110011001100110011001110110011					GGCAGCAGAGCCCGGGGATTCTTC (FAM)
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2531MIRU 2353CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC4348MIRU 3953CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT (NED)Mix 72059MIRU 2077TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA2687MIRU 2454CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)3007MIRU 2753TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)Mix 823474657GCCAGCCGCGTGCATAAACCT (FAM) AGCCACCGGTGTGCCTTCGATCAGT (VIC) CGACGGGCCATCTAGACTACGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC1014954GGTGCGCACCTGCTCCAGATAA (NED) GGTCTCATTGCTGGAGGGTTGTAC	Mix 6	154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT
Image: constraint of the second sec					TACTCGGACGCCGGCTCAAAAT (FAM)
4348MIRU 3953CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCACACAT (NED)Mix 72059MIRU 2077TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA2687MIRU 2454CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)3007MIRU 2753TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)Mix 823474657GCCAGCCGCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCAGTAA (NED)Mix 824614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGCCATCTTGGATCAGCTAC31714954GGTGCGCACCTGCTCCAGATAA (NED)		2531	MIRU 23	53	CTGTCGATGGCCGCAACAAAACG (VIC)
Image: series of the series					AGCTCAACGGGTTCGCCCTTTTGTC
Mix 72059MIRU 2077TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA2687MIRU 2454CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)3007MIRU 2753TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)Mix 823474657GCCAGCCGCCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCATCAGT (VIC)24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC31714954GGTGCGCACCTGCTCCAGATAA (NED)		4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC
Image: Constraint of the constra					CGGAAACGTCTACGCCCCACACAT (NED)
2687MIRU 2454CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)3007MIRU 2753TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)Mix 823474657GCCAGCCGCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC1114954GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC	Mix 7	2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG (FAM)
Image: constraint of the state of the sta					GGAGACCGCGACCAGGTACTTGTA
Image: constraint of the state of the sta		2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT
Mix 823474657GCCAGCCGCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC10131714954GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC					GGGCGAGTTGAGCTCACAGAA (VIC)
Mix 823474657GCCAGCCGCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC31714954GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC		3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA
AGCCACCCGGTGTGCCTTGTATGAC24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC31714954GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC					GCGATGTGAGCGTGCCACTCAA (NED)
AGCCACCCGGTGTGCCTTGTATGAC24614857ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC31714954GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC	Mix 8	2347	46	57	GCCAGCCGCCGTGCATAAACCT (FAM)
3171 49 54 GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC					
3171 49 54 GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC		2461	48	57	
GGCTCTCATTGCTGGAGGGTTGTAC					CGACGGGCCATCTTGGATCAGCTAC
		3171	49	54	
	an 1			1	

4.2 PCR primer sequences

^a Based on the allelic diversity of the MIRU loci observed in *M. tuberculosis*

^b locus 4 in clinical isolates contains an additional invariable MIRU of 53 bp in terminal position of the repeat array. Locus 4052 (QUB-26) may display limited variations in repeat unit length [°] Compared to Supply et al. 2001, *J. Clin. Microbiol.*, 39, 3563-3571, HEX labeling has been replaced by VIC labeling

4.3 Reagent and material references

Reagent/Material	Manufacturer	Reference	Packaging
ART 10 Pipet Tips	ART	2139	10 trays
ART 200 Pipet Tips	ART	2069	10 trays
ART 1000 PipetTips	ART	2079E	8 trays
Thermo-Fast 96, non- skirted plates	ABGENE	AB-0600	25 plates
Adhesive PCR Film	ABGENE	AB-0558	100 sheets
Hot Star Taq Polymerase	QIAGEN	203205	1000U
DNTP set	ROCHE	1969064	4x25 µMol
Unlabeled Oligonucleotides	Eurogentec	OliGold	20 nmol
FAM-, VIC-, NED- labeled oligonucleotides (lyophilized)	ABI	450007	10 nmol
Mapmarker1000-Rox	EUROGENTEC	MW-0195- 80Rox	400 µl
PCR 20 and 100 bp low ladder set	SIGMA	D-7808	25 µg each
FG,Hi-Di Formamide	ABI	4311320	25 ml
3100 POP-4	ABI	4316355	7 ml
Semi-Skirted PCR Plates	SORENSON	35800	25 plates/ pack

4.4 Allele Calling table (top) and H37Rv/Ra genotype (bottom) using the 24

standard MIRU-VNTR loci.

Note: predicted allelic sizes below are those observed using agarose gel electrophoresis. **Offsets MUST be applied to these sizes when using capillary electrophoresis (see text).**

×

H3,	ne ne ne se	(1) 21 22 22 22 22 26 26 4 4 4 4 4 4 4 4 4 4 4
Louis Convention H37Ro-Ra genotype	***	
	8388	MIDRO 4 1 1 M 10 1 M 10
MIRU 02 MIRU 16 MIRU 16 MIRU 20 MIRU 23 MIRU 24 MIRU 26 MIRU 27 MIRU 31 MIRU 39 MIRU 40 VNTR 42 VNTR 44 VNTR 21636 ETRA 154 580 960 1644 2059 2531 2667 296 3007 3192 4348 802 424 577 1955 2165 2165 2 3 3 2 2 6 1 3 3 3 3 2 1 2 4 2 57	Ż	300 Hill 1994 - 1995 -
4 MIRU 10 960 3		MIRROT M Seat 4667 5377 5377 5377 5377 5377 5377 5377 5
- MIRU 16 1644	Ì	1988 1987 1987 1987 1987 1987 1988 1988
MIRU 20 2059 2		No. 1997 No.
MIRU 23 2511 6	***	800 C C C C C C C C C C C C C C C C C C
2687 1		MUNCTRA 3467 3467 3467 3467 3467 3467 3467 3467
MIRU 26 2996		MINULATION (1997)
MIRU 27 3007		 MINULAL MINULAL
MIRU 31 1 3192 3		2 MIR 031 102 200 200 200 200 200 200 200 200 20
4348 2		1 MINO 99 AND
MIRU 40 V 802		
VNTR 42 V 424 2		10000000000000000000000000000000000000
577 4		MILLO14 VARIDA 4 STR MC 4.01 5.77 MC 5.01 7.01 MC 6.02 2.04 MC 6.02 3.04 MC 5.04 5.04 MC 5.0
NTR 1955 1955		
VNTR 216 2163 5		Contraction of the second seco
36 ETRA 316 3		
2347 4		6000 6000 6000 6000 6000 6000 8000 8000
2401 2		
2461 3		Vertic,41 Vertic,47 2047 3401 2047 3401 2048 342 2049 340 2049 341 2049 341 2049 342 2049 342 2049 342 2049 343<
VNTR 49 3171		
VUNTR 3690 3690		
VNIR 46 VNIR 47 VNIR 48 VNIR 49 VNIR 3690 QUB-26 VNIR 55 2347 2401 2461 3171 3690 4052 4156 4 2 3 3 5 5 2		第二日本
VNTR 53 4156 2		ā.
		4114 4114 814 815 815 815 815 815 815 815 815 815 815

4.5 Alternative PCR conditions for Mix 5

The table below includes alternative PCR conditions that can reduce stutter peaks observed with large alleles of locus 4052, especially in multiplex PCR. In this case, the Multiplex PCR kit (Qiagen, Hilden, Germany) is used with DMSO, and NOT the Hotstart Qiagen kit with Q solution.

mix	5
Loci	2163b- 1955- 4052
H2O	4,6
Multiplex PCR	10
Master Mix 2 X	
DMSO	1
Primers EACH ^a	0,4
Total premix	18

Volumes (µl) for the multiplex premix of Mix 5

^a Six in total, *i.e.* one forward and one reverse primer for each of the 3 pairs. Initial concentration of 14 pmol/µl for unlabeled and labeled primers for locus 2163b; 4 pmol/µl for unlabled and labeled primers of locus 1955; 50 pmol/µl for unlabled and labeled primers of locus 4052. See appendix 2 for primer sequence and labeling.