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PROTOCOL BOOK

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Milieu Intérieur
Vers une médecine personnalisée

international network Institut Pasteur

Cepheid.

The Union
International Union Against Tuberculosis and Lung Disease

biocentric
AUTREMENT RÉACTIFS

Multilocus Variable Number Tandem Repeat Genotyping of *Mycobacterium tuberculosis*

Adapted from Multilocus Variable Number Tandem Repeat Genotyping of *Mycobacterium tuberculosis,* Technical guide*, Version 7,* May 2005,

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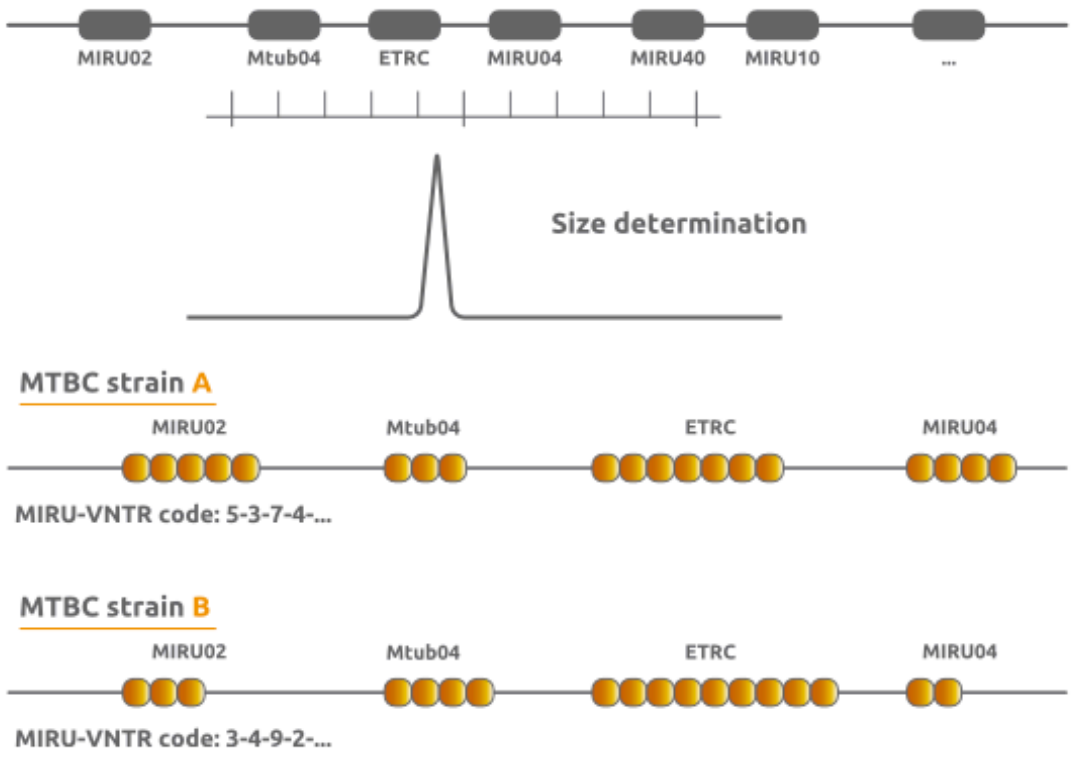


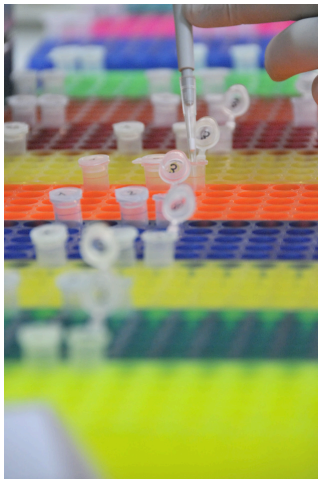
Illustration from Applied Maths, BioNumerics applicationsIntroduction

Mycobacterial Interspersed Repetitive Units (MIRU) - Variable Number Tandem Repeat (VNTR) genotyping relies 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.

For Mycobacterium tuberculosis complex (MTBC) species, multi-locus variable number tandem repeat analysis (MLVA) targeting mycobacterial interspersed repetitive units (MIRU) has been internationally adopted as the new standard, portable, reproducible and discriminatory typing method. (1-8). The freely accessible web application MIRU-VNTRplus (http://www.miru-vntrplus.org) (9, 10) is a bioinformatic web tool available for analysing genotyping data of strains alone or in comparison with a reference database of strains representing the major MTBC lineages

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 (11).

Here is presented the discriminatory subset of 15 loci with the highest evolutionary rates proposed as the MIRU standard for routine epidemiological discrimination of *M. tuberculosis* isolates (12), using simpler electrophoresis with agarose gels.



1. Methods

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

1. DNA extraction and dilution

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.

Procedure

1. Put 200 µl 10 mM Tris-HCl - 1 mM EDTA (pH 7.0) in a screwed cap tube
2. For mycobacteria grown on solid media, take a loop of colonies and resuspend it into a labeled 1.5 ml screw cap tube in 200 µl of 10 mM Tris-HCl, 1 mM EDTA (pH 7.0)
3. For liquid cultures, centrifuge 1,5 mL at 10,000 g x 5 min, in a standard table top centrifuge with aerosol tight rotor, discard the supernatant, wash pellet twice with 1 ml of TE centrifuge at 10,000 rpm x 5 min, discard the supernatant, resuspend the bacterial pellet into 200 µl 10 mM Tris-HCl, 1 mM EDTA (pH 7.0).
4. Incubate at 95°C for 15 min, using an oven or a PCR cycler with a hot lid, if available in the microbiological security facility.
5. Centrifuge the suspension at 10,000 rpm x 1 min, to pellet the cell debris.
6. Harvest the supernatant containing the DNA and transfer into a new tube.
7. Store concentrated stocks at –20 °C until further use.

2. In vitro amplification of the VNTR by PCR

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 only electrophoresis with agarose gels, separate amplification of each locus is performed, using unlabeled oligonucleotides.

Carefully read the procedure « Amplification set up » and rigorously follow it to avoid contaminations

Procedure

1. Preparation of a PCR spreadsheet

- Prepare a PCR spreadsheet, indicating the position of each sample in the PCR 96-well microplate (Figure 1, 2).
- If different 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.
- Include positions for a negative control (sterile water) and a positive control (H37Rv or BCG Pasteur) for each set, to validate the analysis.

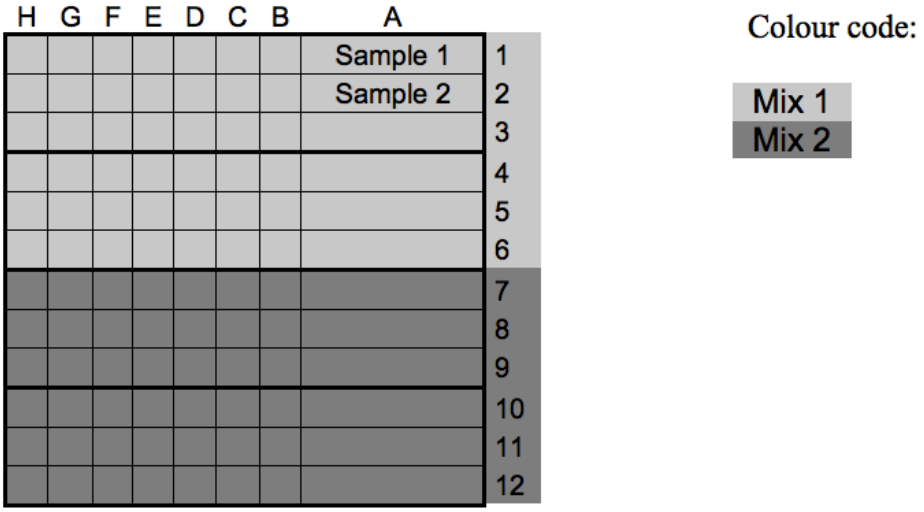


Figure 1. PCR spreadsheet model

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 $MgCl_2$ varies from 1.5 mM (default concentration using the 10 X buffer) to 3 mM (by including additional $MgCl_2$), according to the multiplex or the MIRU locus. The final volume per reaction (after addition of DNA) is 20 µl.

The use of Qiagen Hotstart Taq Polymerase kit including Q 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.

1) In a DNA-free area, prepare the PCR reaction premixes for the different reactions, according to Table 1. 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 as shown in Figure 2 or plates of different colors
3) Dispense 18 µl of the PCR premix into each well of the microplate.

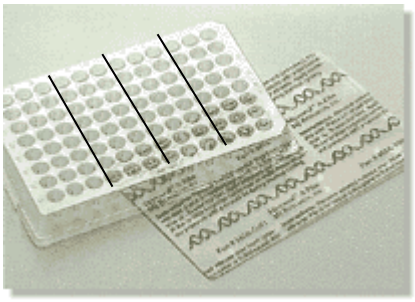


Figure 2. Delineation of different zones on PCR microplate

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 (20 µM)	0,4	0,4	0,4	0,4
Forward primer (20 µM)	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 1. Volumes (µl) for PCR premixes

3. Addition of genomic DNA

4) In another PCR product-free area, dispense 2 µl of extracted DNA 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.
5) Tightly seal the PCR microplate with caps or using an adhesive PCR film, to prevent evaporation during amplification.

4. PCR amplification

6) Amplify the target loci using the PCR conditions indicated in **Erreur ! Source du renvoi introuvable.**. These conditions have been successfully tested on Hybaid PCR express and Perkin Elmer cyclers.
7) Store the PCR products at 4°C or -20°C until further use.

15 min	95°C	
1 min	94°C	40 cycles
1 min	59°C	
1 min 30s	72°C	
10 min	72°C	
∞	4°C	

Table 2. PCR cycling conditions

3. PCR product analysis using agarose gel electrophoresis

Principle

When each locus is amplified separately (i.e. by simplex PCR), the amplified fragments can be analyzed by electrophoresis using agarose gels. (See Agarose gel electrophoresis protocol). 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.

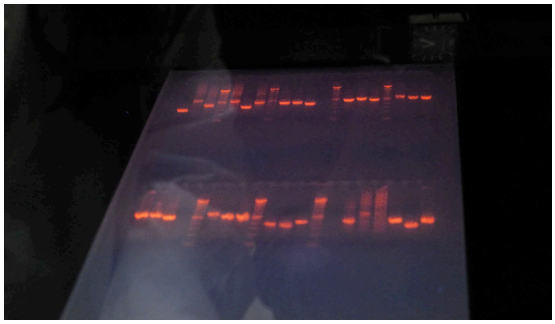
This method is also used to size PCR fragments from reference strains, (in addition to H37Rv), selected to sample the allelic range of each MIRU-VNTR locus. When labeled with fluorescent dyes, the corresponding reference PCR products can then be used to calibrate the sizing using electrophoresis with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

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.

Procedure

1. Gel preparation and electrophoresis

1) Prepare a 3 % suspension of Nu-Sieve agarose gel in 1 x TAE 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, add BET and cast a 25-cm gel, using a shark tooth comb.
4) After solidification, place the gel into an electrophoresis tank containing 1 X TAE.
5) Load 10 µl of a 100-bp ladder size standard marker in both external wells and in the central well 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 5 µl of PCR product with 2 µl of loading buffer. Migrate amplicons from a given locus together on a same gel, 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
7) Run at 120 V for 3-4 hours (or at 20V overnight about15h)
8) Expose the gel to UV light and take a photo.



2. Sizing and allele assignation

Most often, amplification results in single sharp PCR products (**Figure 4**).

1) Determine the size of the sharp PCR product by comparison with the position of the size standard marker.

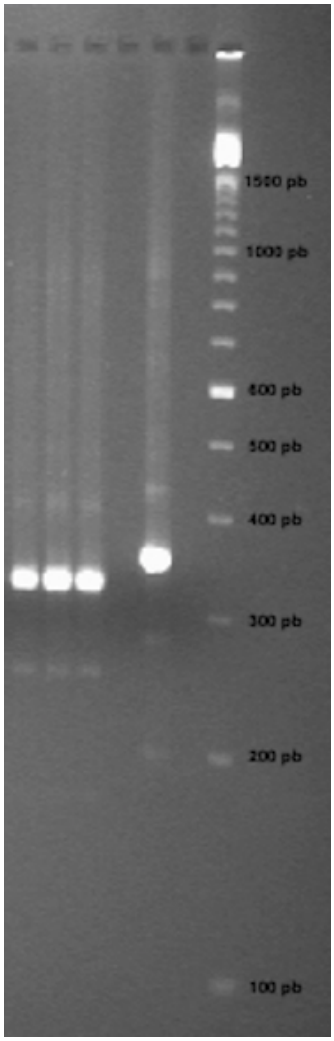


Figure 3. Standard molecular weight marker

2) Determine the corresponding repeat number, using the provided table containing the allele calling for each VNTR locus (Table 3). Verify the consistence with the usual allelic range.

Table 3. Number of MIRU-VNTR repetitions depending on the size of amplicons (bp)

Allele	MIRU 04*	MIRU 10	MIRU 16	MIRU 26	MIRU 31	MIRU 40	VNTR 424	VNTR 577	VNTR 1955	VNTR 2163b	VNTR 2165	VNTR 2401	VNTR 3690	VNTR 4052	VNTR 4156
0	175	482	565	285	492	354	537	171	92	77	195	247	272	177	563
1	252	537	618	336	545	408	588	208	149	146	270	305	330	288	622
2	329	590	671	387	598	462	639	266	206	215	345	363	388	399	681
3	406	643	724	438	651	516	690	324	263	284	420	421	446	510	740
4	483	696	777	489	704	570	741	382	320	353	495	479	504	621	799
5	560	749	830	540	757	624	792	440	377	422	570	537	562	732	858
6	637	802	883	591	810	678	843	498	434	491	645	595	620	843	917
7	714	855	936	642	863	732	894	556	491	560	720	653	678	954	976
8	791	908	989	693	916	786	945	614	548	629	795	711	736	1065	1035
9	868	961	1042	744	969	840	996	672	605	698	870	769	794	1176	1094
10	945	1014	1095	795	1022	894	1047	730	662	767	945	827	852	1287	1153
11	1022	1067	1148	846	1075	948	1098	788	719	836	1020	885	910	1398	1212
12	1099	1120	1201	897	1128	1002	1149	846	776	905	1095	943	968	1509	1271
13	1176	1173	1254	948	1181	1056	1200	904	833	974	1170	1001	1026	1620	1330
14	1253	1226	1307	999	1234	1110	1251	962	890	1043	1245	1059	1084	1731	1389
15	1330	1279	1360	1050	1287	1164	1302	1020	947	1112	1320	1117	1142	1842	1448

* Number of MIRU-VNTR repetitions specific to locus 04

(rare alleles from strains H37Rv, H37Ra, BCG and <1% of clinical strains)

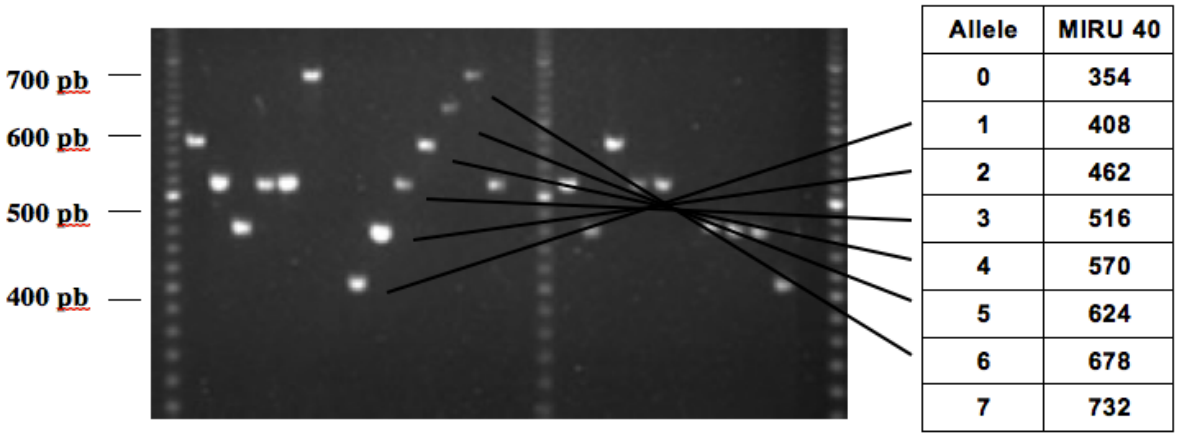
Allele	MIRU 04
0’	122
1’	199
2’	276
3’	353

3. Verify that the allele assignation of the H37Rv control is correct.

Number of repetitions in the control strain H37Rv :

MIRU 04	MIRU 10	MIRU 16	MIRU 26	MIRU 31	MIRU 40	VNTR 424	VNTR 577	VNTR 1955	VNTR 2163b	VNTR 2165	VNTR 2401	VNTR 3690	VNTR 4052	VNTR 4156
3'	3	2	3	3	1	2	4	2	5	3	2	5	5	2

- 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.
- If classical stutter peak ladders 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.



Allele	MIRU 40
0	354
1	408
2	462
3	516
4	570
5	624
6	678
7	732

Figure 4 Example of allele identification for MIRU-VNTR locus 40

4. Detection and interpretation of stutter peaks

Depending on the locus, this sharp amplicon can be accompanied by a ladder of much lower intensity bands, called stutter peaks (Figure 5). 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).

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

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.

Allele	MIRU 27
0	498
1	551
2	604
3	657
4	710
5	763

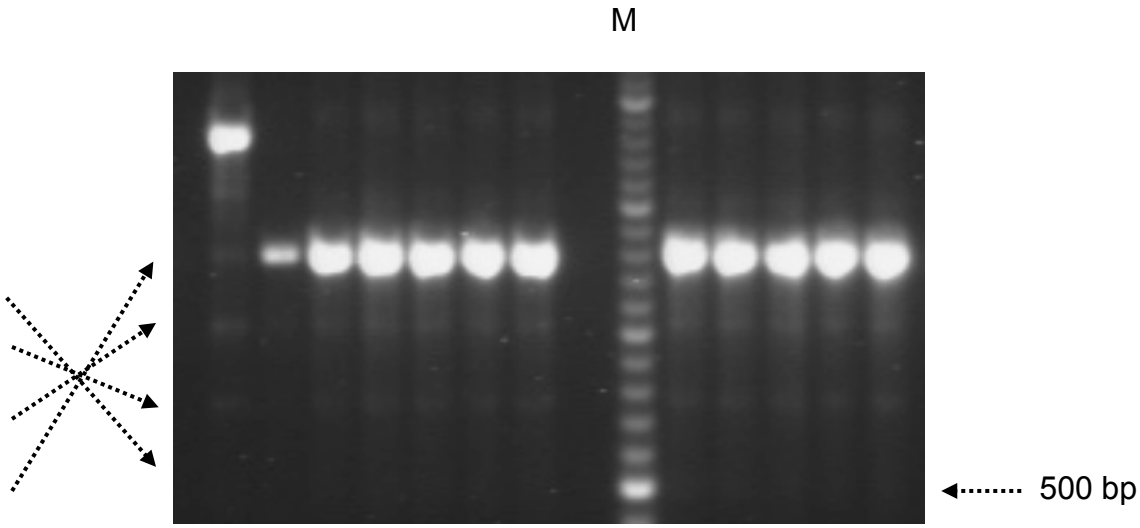


Figure 5 Example of stutter peak detection in a MIRU-VNTR

2. Bioinformatic analysis

http://www.miru-vntrplus.org(9, 10)

Analysis and comparisons of genotypes can be based on MLVA-, spoligotype-, large sequence polymorphism and single nucleotide polymorphism data, or on a weighted combination of these markers. Tools for data exploration include search for similar strains, creation of phylogenetic and minimum spanning trees and mapping of geographic information.

3. Bibliographic references

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SUPPLEMENT 1

Conventional and alias designations of MIRU-VNTR loci

MIRU-VNTR convention[a]	Alias 1	Alias 2
154	MIRU 02	
424	VNTR 42	
577	VNTR 43	ETRC
580	MIRU 04	ETRD
802	MIRU 40	
960	MIRU 10	
1644	MIRU 16	
1955		
2059	MIRU 20	
2163b		QUB-11b
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-4156c
4348	MIRU 39	

[a]MIRU-VNTR loci are listed according to their position in kbp on the H37Rv genome

SUPPLEMENT 2

PCR primer sequences

	Locus	Alias	Repeat unit length (bp)*	PCR primer sequences (5' to 3')
1	580	MIRU 4; ETR D	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC
2	2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
3	802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
4	960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
5	1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
6	3192	MIRU 31; ETR E	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
7	424	Mtub04	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC
8	577	ETR C	58	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACGCGCAAATTGTGA
9	2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
10	2401	Mtub30	58	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA
11	3690	Mtub39	58	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
12	4156	QUB-4156	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
13	2163b	QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
14	1955	Mtub21	57	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
15	4052	QUB-26	111	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCGAT

* 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

SUPPLEMENT 3

Supplies

Reagent/Material	Supplier	Order number
ART 10 Pipet Tips	ART	2139
ART 200 Pipet Tips	ART	2069
ART 1000 PipetTips	ART	2079E
Thermo-Fast 96, non-skirted plates	ABGENE	AB-0600
Semi-Skirted PCR Plates	SORENSON	35800
Adhesive PCR Film	ABGENE	AB-0558
Hot Start Taq Polymerase	QIAGEN	203205
dNTP set 100mM	ThermoFisher	10297018
Unlabeled Oligonucleotides	Sigma	
100pb Ladder	ThermoFisher	15628019
NueSieve Agarose 3:1	TEBU	50091
NueSieve Agarose 3:1 Loading blue buffer	TEBU	50090