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Rev.	Descrizione modifiche	Data
0	Prima emissione	12/01/2011
1	Inserite Quadruplex e aggiornamenti software di interpretazione	06/08/2012

Compilazione	Sviluppo	Verifica	Approvazione
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1. SCOPE

This protocol describes all the steps and information needed to perform 24-locus VNTR typing of *M. tuberculosis* complex isolates by two different methods:
- Method 1 (in-house): this method can be performed as eight multiplex PCRs and analysis of the PCR product sizes on an automated sequencer or as 24 single PCRs and analysis of the PCR product sizes on agarose gel.
- Method 2 (automated): A commercial kit by which analysis of the PCR fragment sizes is possible by using an automated sequencer.

2. APPLICATION

The present instruction is applicable within the mycobacterial typing area (TM) and within the mycobacterial research area.

3. DEFINITIONS AND ABBREVIATIONS

VNTR	Variable Number of Tandem Repeats
MIRU	Mycobacterial Interspersed Repetitive Unit
ETR	Exact Tandem Repeat
QUB	Queens University of Belfast
MQ	MilliQ
RT	Room Temperature
DNA	Desoxyribo Nucleic Acid
Rpm	Rotations per minute
TE	Tris EDTA

4. RESPONSIBILITIES

The supervision and the correct application of the following instruction is a responsibility of the area coordinator. The execution of the procedure is responsibility of area technicians, master students and coordinator.

5. EQUIPMENT AND MATERIALS

- 96-well plate
- Multichannel micro pipette
- Adhesive transparent film
- Centrifuge for 96-well plate
- Thermal cycler
- Personal protection equipment (lab coat, gloves, goggles).
- Pipettes and adapted tips.
- Vortex mixer.
- Microwave oven
- Electrophoresis system for at least 25-cm gel
- Gel picture capture system
- Applied Biosystems 3730/3730 XL DNA Analyzer.
- GeneMapper version 3.7.
- 0.2 ml tubes, strips or 96-well plate for PCR amplification.
- 100-bp and 50-bp or 20-bp size standard.
- NuSieve Agarose 3:1
- Tris/Borate/EDTA (TBE) buffer solution
- Ethidium bromide or DNA staining compounds (Gel Red)
- For analysis of the PCR fragments on a DNA sequencer; GeneScan LIZ1200 size standard

(Applied Biosystems, Foster City, CA)
- 24 primer-sets (Annex 3): labeled primers are only necessary for the detection of amplicon sizes by an automated sequencer.
- HotStarTaq DNA Polymerase kit (QIAGEN, Hilden, Germany)
  Components: HotStarTaq DNA Polymerase, PCR buffer (10x), Q-Solution (5x) and MgCl2 (25 mM).
- dNTP's PCR Grade; prepare 10 mM dNTP work solution and store in small portions at -20℃.
- MIRU-VNTR Typing Kit (GenoScreen, Lille, France).
  Components: PCR mix 1-6, Positive control, store at -20℃ (avoid multiple freeze-thaw cycles)
- Software GeneMapper 3.7 or updated versions (4.0; 4.1) - (Applied Biosystems, Foster City, CA)

6. PROCEDURES

6.1 Principle of the Procedure

Variable Numbers of Tandem Repeats (VNTR) sequences have emerged as valuable markers for genotyping of various bacterial species. VNTR typing makes use of the variability in the numbers of repeats present at particular known tandem repeat loci in bacterial genomes. The methodology comprises PCR amplification using primers specific for the regions flanking tandem repeat loci and the determination of the sizes of the amplicons, after electrophoretic migration. As the length of the repeat units is known, the sizes of the PCR products reflect the number of repeats in each VNTR locus. The final result is a numerical code, corresponding to the number of tandem repeats present in each VNTR locus and this serves as a DNA fingerprint of a bacterial isolate.
For a long time, IS*6110* restriction fragment length polymorphism (RFLP) typing was used to study the epidemiology of *Mycobacterium tuberculosis* complex, however, since 2006 VNTR typing is the internationally recommended typing method for *M. tuberculosis* (Supply et al. 2006). Both IS*6110* RFLP and VNTR typing are highly discriminative and reproducible methods for typing of *M. tuberculosis* (Kremer et al. 2005). However, compared to IS*6110* RFLP typing, VNTR typing has several advantages; it has a much shorter turn-around time and is appropriate for all *M. tuberculosis* complex isolates, including strains that have a few IS*6110* copies, and, most importantly, it can be applied to very little amounts of DNA. Although also this technique is still technically demanding, it is simpler to perform than RFLP typing.
The length of the PCR products with tandem repeats can be determined on classical agarose gels, providing appropriate markers are used. However, also a high throughput VNTR genotyping method has been developed, which combines multiplex PCRs, analysis of the combined PCR fragments on a fluorescence-based DNA analyzer, and automated allele calling (Supply et al. 2002). For the international standardization, a subset of 24 loci was selected to provide an appropriate balance between variability among closely related but epidemiologically unlinked isolates and stability among pre-identified epidemiologically linked isolates (Supply et al. 2006). These 24 loci comprise tandem repeat loci which are also known as mycobacterial interspersed repetitive units (MIRUs), Exact Tandem Repeats (ETRs), and Queens' University of Belfast (QUB) loci, because of their nature or place of discovery.

6.2 Preparation of the PCR Mix

Method 1 (Single locus amplification)

• Calculate the amount of reagents needed for the PCR (Annex 1)
• Print the PCR mix schedule.
• Prepare the different PCR reaction mixes in an amplicon-free PCR clean room, by combining the reagents mentioned in the PCR-mix schedule (MQ or sterile water, PCR buffer, Q-Solution, MgCl2, mix specific primers and HotStarTaq polymerase). Primers' stock solutions must be diluted 1:10 in order to obtain a working solution of primers 10 µM.
• Fill out the mixes following your personal pipette plan. Add 45 µl PCR mix to each well (or tube).

• In a DNA dedicated area, add 5 µl of DNA solutions. Total reaction volume is 50 µl.

Method 2 (Automated)

• Soon after the receipt of the Kit, containing 6 Quadruplex mixes (Annex 2), aliquot the primers (it is possible to dilute each triplex 2:3 with sterile water or MilliQ in a 2.0 ml Eppendorf tube).
• Add **64 µl** to the first line (A) according to the following scheme. The first two columns with Qualdruplex 1, the third and the fourth with the Qualdruplex 2 and so on.

	Q1	Q2	Q3	Q4	Q5	Q6	Q1	Q2	Q3	Q4	Q5	Q6
	1	2	3	4	5	6	7	8	9	10	11	12
A	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl	64 µl
B												
C												
D												
E												
F												
G												
H												

• By a multichannel micropipette, transfer **8 µl** of each Quadruplex by dispensing vertically from the line A to line H in order to have 8 µl in each well at the end of the procedure.
• Cover the plate with an adhesive film.
• Spin the 96-well plate and store it -20°C sealed in aluminum foil by marking it with the appropriate lot and plate number. (i.e. MV-1 #4: lot number MV1, plate number 4).
• Before loading DNA samples, spin down the 96-well plate
• Load **2 µl** of DNA solutions following this order:

	Q1	Q2	Q3	Q4	Q5	Q6	Q1	Q2	Q3	Q4	Q5	Q6
	1	2	3	4	5	6	7	8	9	10	11	12
A	ID1	ID1	ID1	ID1	ID1	ID1	ID9	ID9	ID9	ID9	ID9	ID9
B	ID2	ID2	ID2	ID2	ID2	ID2	ID10	ID10	ID10	ID10	ID10	ID10
C	ID3	ID3	ID3	ID3	ID3	ID3	ID11	ID11	ID11	ID11	ID11	ID11
D	ID4	ID4	ID4	ID4	ID4	ID4	ID12	ID12	ID12	ID12	ID12	ID12
E	ID5	ID5	ID5	ID5	ID5	ID5	ID13	ID13	ID13	ID13	ID13	ID13
F	ID6	ID6	ID6	ID6	ID6	ID6	ID14	ID14	ID14	ID14	ID14	ID14
G	ID7	ID7	ID7	ID7	ID7	ID7	PC	PC	PC	PC	PC	PC
H	ID8	ID8	ID8	ID8	ID8	ID8	NC	NC	NC	NC	NC	NC

PC: Positive Control (BCG, H37Rv)
NC: Negative Control (MilliQ, sterile water)

• Cover the plate with an adhesive film.
• Spin down the 96-well plate to remove air bubbles
• Put the plate containing PCR mix and DNA into the thermal cycler

6.3 PCR Programmes

Method 1 (Manual)

15:00 95°C
1:00 95°C
1:00 59°C } x 30 o x 40
1:30 72°C

10:00 72°C
∞ 4°C

Method 2 (Automated)

15:00	95°C	
1:00	94°C	40x
0:30	59°C	40x
1:30	72°C	40x
10:00	72°C	
∞	4°C	

6.4 Sample Preparation for Automated Fragment Analysis (Method 2 - Sequencer)

• Spin down the sample plate containing the PCR products.

• Dilute the LIZ-marker 20 times by adding 50 µl of 1200LIZ marker to 950 µl HiDi Formamide or MQ in a tube.

• Fill out 10 µl diluted LIZ-marker in each well of a new 96-well plate marking it with the appropriate lot and plate number (the same as the PCR plate)

• Add **2 µl** of the PCR-products to the plate with LIZ-marker.

• Close the plate with an adhesive film

• Close the original sample plate also, and store this plate in the freezer (-20ºC).

Note: In case of problems, the PCR products can be used again.
Note: Fill empty wells in your plate with 10 µl of MQ.

• Centrifuge the plate to remove air bubbles.

• Denaturate the PCR products at 95°C for 5 minutes and keep on ice before loading.

• Remove the adhesive film and cover the plate for sequencing with Plate Septa 96-well.

At the sequencer

• Prepare the worksheet according to the instrument instructions and name the sequencer run with the lot, plate number and date (i.e. MV-1#4_02_04)

• Start the fragment analysis as described in the sequencer manual.

6.5 Sample Preparation for Fragment Analysis by Agarose Gel

• Prepare a 3% Nusieve 3:1 agarose gel.

• Centrifuge the plate/tubes with PCR products to remove air bubbles.

• Load into the gel a base-ladder with DNA fragments which allow you to interpret the fragment lengths of the amplified samples (50 bp or 100 bp range)

• Add **5-10 µl** PCR product to the gel.

• Run the gel with the settings compatible to the used system. 5-6 hours at 100-120 voltage.

• After running the gel, stain the DNA for 30 minutes on a shaking platform and take a picture of the separated PCR products.

• Interpret PCR products sizes according to the table annexed (Annex 4)

7. RECORDING AND REPORTING

After the interpretation by the GeneMapper Software (v 3.7, v 4.0 or v 4.1), results should be saved in the appropriate folder called with the name of the plate run and the date of examination. Files coming from GeneMapper should be copied into the "Mod. EBP-TM 006 – GeneMapper Analysis" in order to be printed out and checked.
Also, results should be reported into the appropriate excel sheet for a specific year or into the appropriate database. Results from agarose gel analysis should be reported into the appropriate excel sheet.

8. RELATED DOCUMENTS

- Frothingham, R., and W. A. Meeker-O'Connell. 1998. Genetic diversity in *the Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144:1189- 1196.
- Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply. 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc. Natl. Acad. Sci. U S A 98:1901-1906.
- Goyal, M., D. Young, Y. Zhang, P. A. Jenkins, and R. J. Shaw 1994. PCR amplification of variable sequence upstream of *katG* gene to subdivide strains of *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. 32:3070
- Magdalena, J., P. Supply, and C. Locht 1998a. Specific differentiation between *Mycobacterium bovis* BCG and virulent strains of the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. 36:2471-2476.
- Magdalena, J., A. Vachee, P. Supply, and C. Locht 1998b. Identification of a new DNA region specific for members of Mycobacterium tuberculosis complex. J. Clin. Microbiol. 36:937-943. 6 8
- Kremer, K., D. van Soolingen, R. Frothingham, W. H. Haas, P. W. Hermans, C. Martin, P. Palittapongarnpim, B. B. Plikaytis, L. W. Riley, M. A. Yakrus, J. M. Musser, and J. D. van Embden. 1999. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J. Clin. Microbiol. 37:2607-2618.
- Roring, S., A. Scott, D. Brittain, I. Walker, G. Hewinson, S. Neill, and R. Skuce. 2002. Development of variablenumber tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J. Clin. Microbiol. 40:2126-2133.
- Skuce, R. A., T. P. McCorry, J. F. McCarroll, S. M. Roring, A. N. Scott, D. Brittain, S. L. Hughes, R. G. Hewinson, and S. D. Neill. 2002. Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. Microbiology. 148:519-528.
- Smittipat, N., and P. Palittapongarnpim. 2000. Identification of possible loci of variable number of tandem repeats in *Mycobacterium tuberculosis*. Tuber. Lung Dis. 80:69-74.
- Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht. 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. 36:762-771.
- Le Fleche, P., M. Fabre, F. Denoeud, J. L. Koeck and G. Vergnaud. 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. BMC Microbiol. 2:37.
- Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht. 2001. Automated High-Throughput Genotyping for Study of Global Epidemiology of *Mycobacterium tuberculosis* Based on Mycobacterial Interspersed Repetitive Units. J. Clin. Microbiol. 39:3563- 3571.
- Supply, P., J. Magdalena, S. Himpens, and C. Locht. 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol. Microbiol. 26:991-1003.
- Steinlein Cowan, L., L. Mosher, L. Diem, J.P. Massey, and J.T. Crawford. 2002. Variable number tandem repeat typing of *Mycobacterium tuberculosis* isolates with low-copy numbers of IS*6110* by using mycobacterial interspersed repetitive units. J. Clin. Microbiol. 40:1592-1602.
- Cowan L.S., L. Diem, T. Monson, P. Wand, D. Temporado, T.V. Oemig, and J.T. Crawford. 2005. Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States. J. Clin. Microbiol. 43:688-695.
- Allix, C., P. Supply, and M. Fauville-Dufaux. 2004. Usefulness of fast mycobacterial interspersed repetitive unitvariable-number tandem repeat typing in clinical mycobacteriology: A study of three cases. Clin. Infect. Dis. 39:783-789
- Hawkey P.M., E.G. Smith, J.T. Evans, *et al.* Mycobacterial interspersed repetitive unit typing of *Mycobacterium*

tuberculosis compared to IS*6110*-based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. 2003. J. Clin. Microbiol. 41:3514-3520

• van Soolingen, D., P. E. de Haas, P. W. Hermans, and J. D. van Embden. 1994. DNA fingerprinting of *Mycobacterium tuberculosis*. Methods Enzymol. 235:196-205.

• GenoScreen MIRU-VNTR Typing Kit, user manual.

• www.tuberculosis.rivm.nl

9. ANNEXES

Annex 1: PCR MIX (Volume are expressed in microliters)

MIX	Protocol A	Protocol B	Protocol C	Protocol D
MilliQ or Sterile water	22.8	24.8	23.8	25.8
10x PCR Buffer (15 mM $MgCl_2$)	5.0	5.0	5.0	5.0
5x Q solution	10.0	10.0	10.0	10.0
$MgCl_2$ 25 mM	3.0	1.0	2.0	---
dNTPs (5 mM each)	2.0	2.0	2.0	2.0
Primer (FW e REV)	1.0	1.0	1.0	1.0
Taq Polymerase 5 U/µl	0.2	0.2	0.2	0.2
Volume µl	45	45	45	45

- Protocol D Loci: MIRU 20, 24, 27, Mtub 04, ETRC, ETRA, Qub-11 b, Mtub 21; Qub-26
- Protocol C Loci: MIRU 2, 23, 39
- Protocol B Loci: MIRU 10, 16, 31, Mtub 29, ETRB, Mtub 34
- Protocol A Loci: MIRU 4, 26, 40, Mtub 30, Mtub 39, Qub-4156

Annex 2: : PRIMER SEQUENCES AND QUADRUPLEX

Locus	Alias	Quadruplex	Lunghezza della ripetizione (pb)	Sequenza 5' – 3' (indicati tra parentesi i fluorofori legati al primer)
580	MIRU 4	1	77	GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGCCAGC
2996	MIRU 26	1	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)
802	MIRU 40	1	54	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	2	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT (FAM)
1644	MIRU 16	2	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (VIC)
3192	MIRU 31	2	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)
424	Mtub04	3	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC (FAM)
577	ETR C	3	58	CGAGAGTGGCAGTGGCGGTTATCT (VIC) AATGACTTGAACGCGCAAATTGTGA
2165	ETR A	3	75	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT
2401	Mtub30	4	58	CTTGAAGCCCCGGTCTCATCTGT (FAM) ACTTGAACCCCCACGCCCATTAGTA
3690	Mtub39	6	58	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
4156	Qub4156	5	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (NED)
2163b	QUB-11b	2	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT (FAM)

1955	Mtub21	1	57	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA
4052	QUB-26	3	111	AACGCTCAGCTGTCGGAT (NED) CGGCCGTGCCGGCCAGGTCCTTCCCGAT
154	MIRU 2	4	53	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)
2531	MIRU 23	4	53	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC
4348	MIRU 39	4	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT (NED)
2059	MIRU 20	5	77	TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA
2687	MIRU 24	5	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)
3007	MIRU 27	5	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)
2347	Mtub29	6	57	GCCAGCCGCCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC
2461	ETR B	6	57	ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC
3171	Mtub34	6	54	GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC

ANNEX 3: INTERPRETATION TABLE

Allele	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
0	402	537	171	175	354	482	565	92	437	77	195	335	247	347	150	395	285	498	326	492	272	187	563	540
1	455	588	208	252	408	537	618	149	514	146	270	392	305	404	200	447	336	551	380	545	330	298	622	593
2	508	639	266	329	462	590	671	206	591	215	345	449	363	461	253	501	387	604	434	598	388	409	681	646
3	561	690	324	406	516	643	724	263	668	284	420	506	421	518	306	555	438	657	488	651	446	520	740	699
4	614	741	382	483	570	696	777	320	745	353	495	563	479	575	359	609	489	710	542	704	504	631	799	752
5	667	792	440	560	624	749	830	377	822	422	570	620	537	632	412	663	540	763	596	757	562	742	858	805
6	720	843	498	637	678	802	883	434	899	491	645	677	595	689	465	717	591	816	650	810	620	853	917	858
7	773	894	556	714	732	855	936	491	976	560	720	734	653	746	518	771	642	869	704	863	678	964	976	911
8	826	945	614	791	786	908	989	548	1053	629	795	791	711	803	571	825	693	922	758	916	736	1075	1035	964
9	879	996	672	868	840	961	1042	605	1130	698	870	848	769	860	624	879	744	975	812	969	794	1186	1094	1017
10	932	1047	730	945	894	1014	1095	662	1207	767	945	905	827	917	677	933	795	1028	866	1022	852	1297	1153	1070
11	985	1098	788	1022	948	1067	1148	719	1284	836	1020	962	885	974	730	987	846	1081	920	1075	910	1408	1212	1123
12	1038	1149	846	1099	1002	1120	1201	776	1361	905	1095	1019	943	1031	783	1041	897	1134	974	1128	968	1519	1271	1176
13	1091	1200	904	1176	1056	1173	1254	833	1438	974	1170	1076	1001	1088	836	1095	948	1187	1028	1181	1026	1630	1330	1229
14	1144	1251	962	1253	1110	1226	1307	890	1515	1043	1245	1133	1059	1145	889	1149	999	1240	1082	1234	1084	1741	1389	1282
15	1197	1302	1020	1330	1164	1279	1360	947	1592	1112	1320	1190	1117	1202	942	1203	1050	1293	1136	1287	1142	1852	1448	1335