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PROTOCOL BOOK

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International Union Against Tuberculosis and Lung Disease

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AUTREMENT RÉACTIFS

Spoligotyping

adapted from “SPOLIGOTYPING” a PCR-based method to simultaneously detect and type *Mycobacterium tuberculosis* complex bacteria *Version 1.3 19/04/2016*

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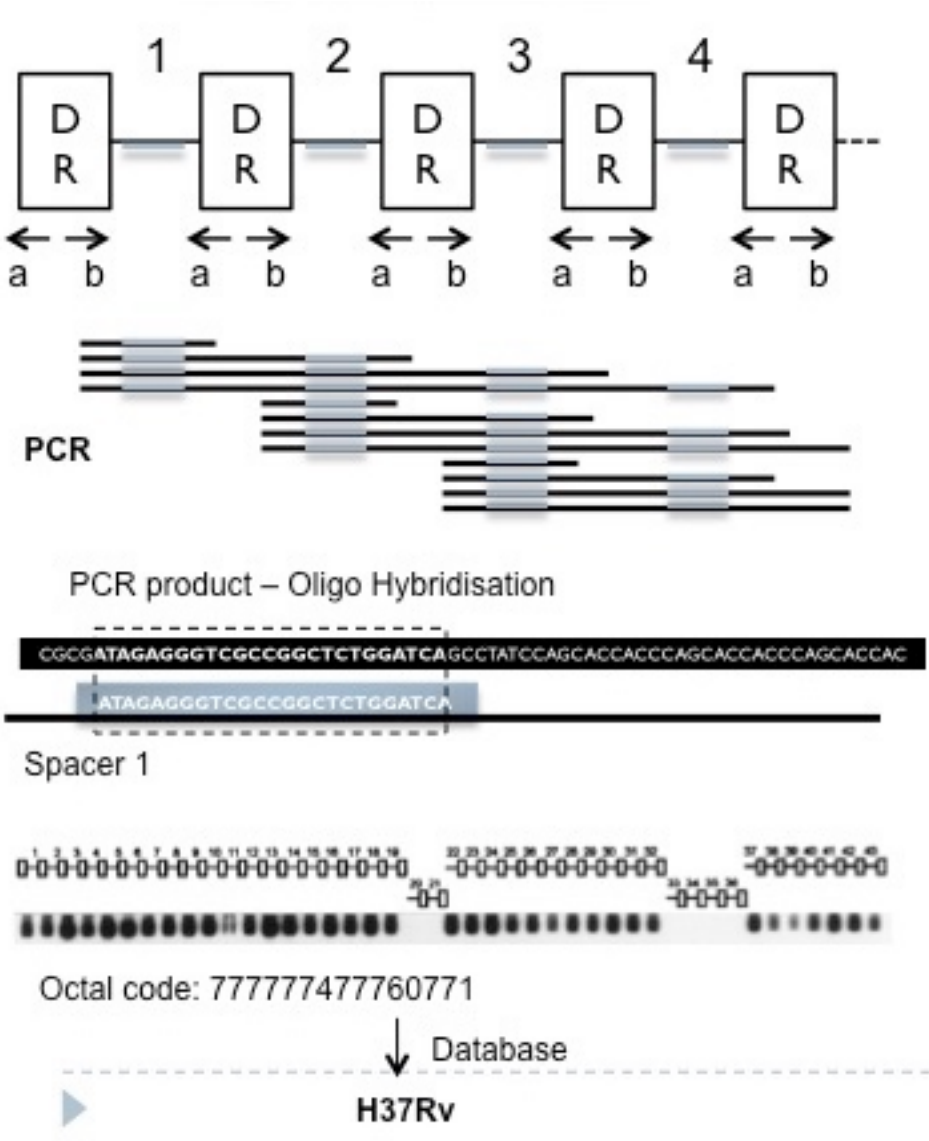


1. General description

1. Principle of the spoligotyping method

The typing method described in this protocol is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria. This locus was first described by Hermans et al.[5] who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. The number of copies of the DR sequence in *M. bovis* BCG was determined to be 49. In other *M. tuberculosis* complex strains the number of DR elements was found to vary significantly. The vast majority of the *Mycobacterium tuberculosis* strains contain one or more IS*6110* elements in the DR region (Fig.1).

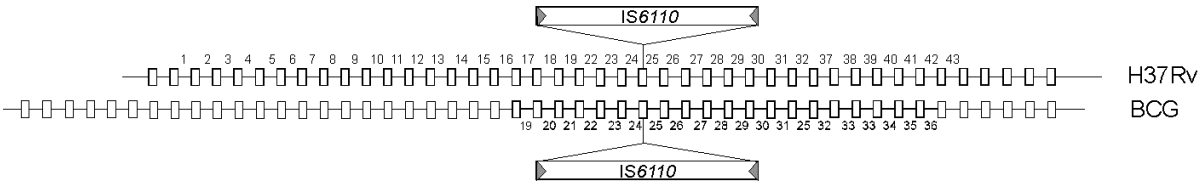


Figure 1. Structure of the DR locus in the genome of *M. tuberculosis* H37Rv and *M. bovis* BCG P3. The rectangles depict the 36 bp Direct Repeat (DR).

In contrast to the DRs, the spacers are usually present only once in the DR region, but occasionally some are found twice, either separated by one or by several DR's and other spacers. One DR and its neighbouring non-repetitive spacer is termed “Direct Variant Repeat” (DVR).

When the DR regions of several strains were compared, it was observed that the order of the spacers is about the same in all strains, but deletions and/or insertions of spacers and DR's occur (Fig. 2)[2]. The mechanism by which spacers and copies of DR are generated, is unknown. With the method described here, the presence or absence in the DR region of 43 spacers of known sequence can be detected by hybridization of PCR-amplified spacer DNA to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences. This method will be referred to as *spoligotyping* (from *sp*acer *oligotyping*)[7].

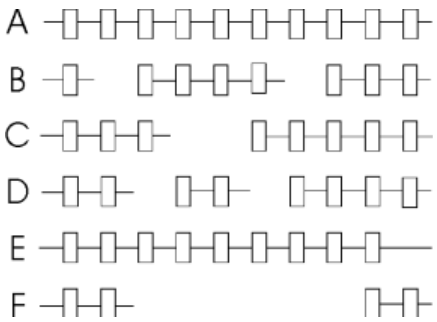


Figure 2. Schematic presentation of the polymorphism in DR regions of different *M. tuberculosis* complex strains. Blocks of DVR are missing in one strain when compaired to another. The spacer order remains about the same

By spoligotyping one can detect the presence or absence of spacers of known sequence. The first step in the method is to amplify the DR region of a given strain by PCR. The primers used are based on the sequence of the DR, and allow the amplification of the spacer(s) between the DR targets (Fig. 3). The obtained PCR products differ in length because of two reasons. First, the product contains several spacers and the DR's in between if the primers anneal to DR's not next to each other. Second, the product itself can act as a primer, and become elongated with one or more DVRs. Therefore, the PCR product provides no reliable information about spacer order or total length of the DR region. A biotin labelled reverse primer is used, so that all the reverse strands synthesized are biotin labelled.

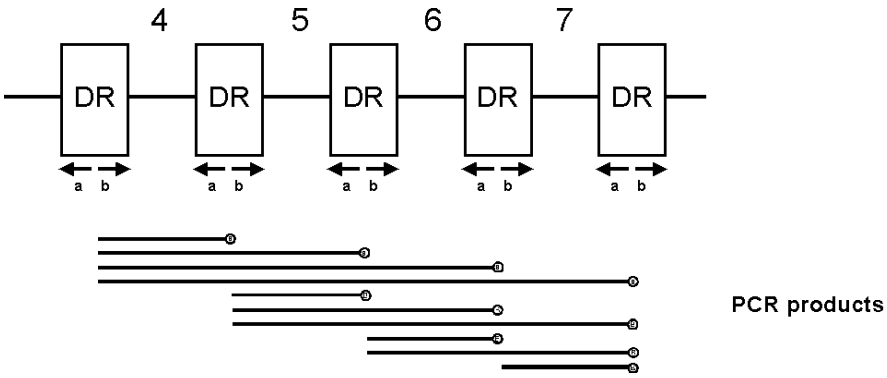


Figure 3. Principle of the *in vitro* amplification of DNA within the DR region of *M. tuberculosis* complex bacteria. The use of the 2 primers, a and b, for *in vitro* amplification, will lead to the amplification of any spacer or a stretch of neighbouring spacers and DR's.

Oligonucleotides derived from the known spacers in the DR cluster are covalently linked to an activated Biodyne C membrane in parallel lines. PCR products are hybridized perpendicular to the oligo lines. After hybridization the membrane is incubated in streptavidin peroxidase, which binds to the biotin label on the PCR products. Detection of hybridization signals is done by the enhanced chemiluminescence (ECL) detection system. The peroxidase present on the streptavidine catalyzes a reaction resulting in the emission of light, which can be

detected by autoradiography of the membrane. We refer to this type of blot as *reversed line blot* (Fig. 4).

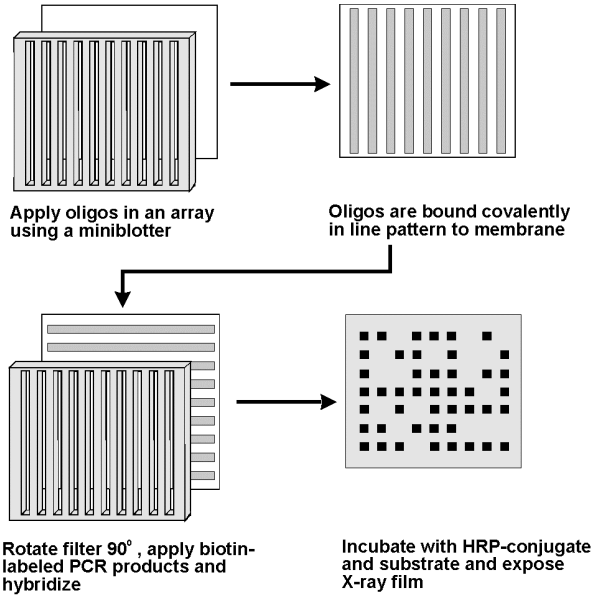


Figure 4. Overview of the spoligotyping metho

An example of a result of the spoligotyping method used to analyze a variety of clinical isolates is shown in Fig. 5 and 6.

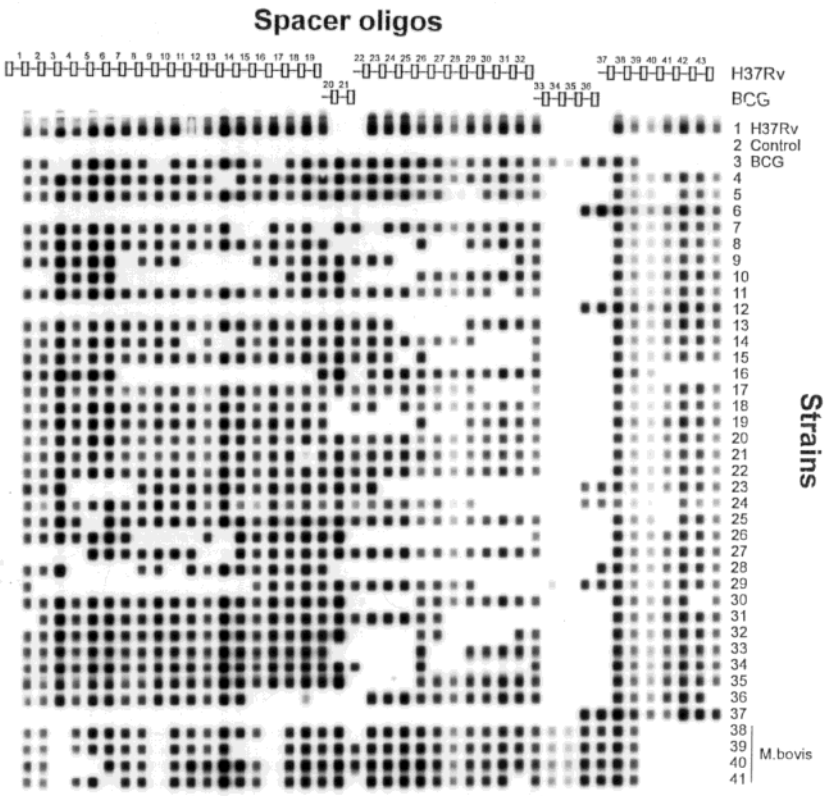


Figure 5. Spoligotyping result of *M. tuberculosis* H37Rv, *M. bovis* BCG P3 and 38 different clinical isolates. A membrane with 43 spacer oligonucleotides was used (vertical lines). The spacer oligonucleotides were derived from the spacers of *M. bovis* BCG P3, *M. tuberculosis* H37Rv.

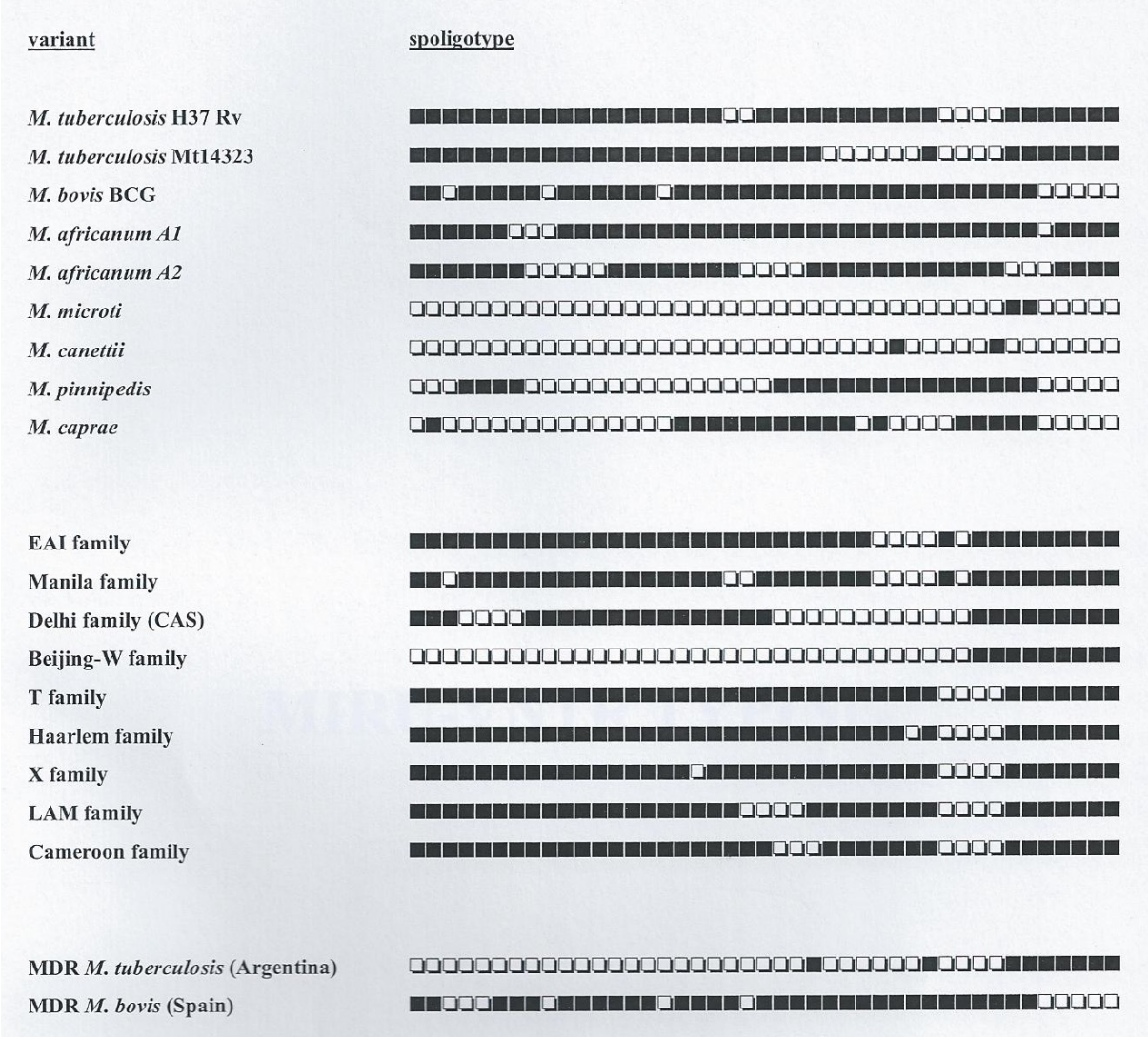


Figure 6. Spoligotyping result of Mycobacterium species

2. Practical use

Spoligotyping may offer an alternative for typing Southern blotting when rapid results are required. The method is in particular useful to simultaneously detect and type *M. tuberculosis* complex bacteria in clinical samples (suspected nosocomial infections, outbreaks in prisons, etc.). The level of differentiation by spoligotyping is less compared to IS*6110* fingerprinting for strains having five or more IS*6110* copies, but higher for strains with less than five copies. Thus spoligotyping is a preferred method to type *M. bovis* strains, which usually contain only one or two IS*6110* copies. Note that *M. bovis* can be recognized by the absence of reactivity with spacers 39-43 (Fig. 5).

2. Methods

1. In vitro amplification of spacer DNA by PCR

Principle

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal Mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Löwenstein) can also serve as template (see supplement 2). The PCR products are labeled with biotin, because one of the primers is biotinylated. The primers for the PCR are based on the DR sequence:

DRa : 5'-GGT TTT GGG TCT GAC GAC-3', biotinylated at 5' end.

DRb : 5'-CCG AGA GGG GAC GGA AAC-3'

Procedure

1) Preparation of lysates from bacteria
   - For bacteria grown on solid media, collect bacteria take a loop of colonies and suspend it into a labeled 1.5 ml screw cap tube in 200 µl of 10 mM Tris-HCl, 1 mM EDTA (pH 7.0)
   - For liquid cultures, centrifuge 1,5 mL at 10000 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 10000 rpm x 5 min, discard the supernatant, resuspend the bacterial pellet into 200 µl 10 mM Tris-HCl, 1 mM EDTA (pH 7.0).
   - Inactivate bacteria at 95°C for 15 min
   - Centrifuge for 5 minutes at 10000 g
   - Keep supernatant containing the DNA and transfer it into a new tube

   Always include DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG as positive controls. Use water as a negative control.

2) Prepare the reaction mixture

   Carefully follow the procedure « Amplification set up » to avoid contaminations

For 1 PCR reaction : 48 µl (+ 2µl sample)		
Reagents	**Final Concentration**	**Quantity (µl)**
10X Buffer	1X	5
$MgCl_2$ (2 mM)	50 mM	2
Primer DRa 5 µM [a)]	0.4 µM	4
primer DRb 5 µM [b)]	0.4 µM	4
dNTP 2,5mM each	200 µM	4
Eurobiotaq (5U/µl)	2,5 U	0.5
H_2O		28,5
Final mix volume		**48**

3) Add 2 µL of lysate
4) Tightly seal the PCR microplate with caps to prevent evaporation during amplification.
5) Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling (Table 1).

Table 1. PCR cycling conditions

3 min	96°C	
1 min	96°C	20 cycles
1 min	55°C	
30 sec	72°C	
5 min	72°C	
∞	4°C	

6) Store the PCR products at 4°C or -20°C until further use.

a) Remarks

- Preparation of the mixture has to take place in a laboratory free of mycobacterial PCR products containing the DR sequences. DNA has to be dispensed in another PCR product-free area.
- Primer DRa is biotinylated and should be stored at **+4°C**. Repeated freeze- thawing of the biotinylated primer results in weaker Spoligopatterns.
- Primer DRb should be stored in small aliquots at -20°C.
- For amplification of the DR-cluster from extracts of clinical samples, the number of cycles can be increased to 40.
- For amplification of the DR-cluster from heat-killed cells, the number of cycles can be increased to 30 (see also supplement 2B, Preparation of lysates from colonies).

2. PCR product analysis using agarose gel electrophoresis

- Optionnal analyze of 5 µL of the PCR product on a 2% agarose gel (see Agarose gel protocol).
- A ladder pattern should be visible.

3. Membrane hybridization with PCR product and detection

a) Purpose

Hybridization of the biotin-labeled PCR products to the immobillized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

b) Note

- All incubations should take place in a plastic container under gentle shaking, unless otherwise stated.
- Thoroughly clean the miniblotter with soap and a dedicated brush before use.
- Never touch the membrane with gloves, the powder causes background. Use foreceps.
- The quality of the SDS is of critical importance. It should be fresh, do not store it for longer than one week. We have good experience with SDS from BDH Laboratory Supplies.

c) Procedure

1) All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):

250 ml 2xSSPE/0.1% SDS, 60ºC,

500 ml 2xSSPE/0.5% SDS, 60ºC,

500 ml 2xSSPE/0.5% SDS, 42ºC.

500 ml 2xSSPE, room temperature.

2) Add 20 µl of the PCR products to 150 µl 2xSSPE/0.1% SDS.
3) Heat-denature the diluted PCR product for 10 min at 99°C and cool on ice immediately.
4) Wash the membrane for 5 min at 60°C in 250 ml 2xSSPE/0.1% SDS.
5) Place the membrane and a supportcushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides. [a)]
6) Remove residual fluid from the slots of the miniblotter by aspiration.
7) Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60ºC on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots. [b)]
8) Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.
9) Wash the membrane twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 60°C .
10) Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.
11) Add 2.5 µl streptavidin-peroxidase conjugate (500U/ml) to 10 ml of 2xSSPE/0.5% SDS, and incubate the membrane in this solution for 45 to 60 min at 42°C in the rolling bottle.
12) Wash the membrane twice in 250 ml of 2xSSPE/0.5% SDS for 10 min at 42°C.
13) Rinse the membrane twice with 250 ml of 2xSSPE for 5 min at room temperature.
14) For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 20 ml ECL detection liquid. [c)]
15) Cover the membrane with a transparant plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 20 min. [d)]
16) If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

d) Remarks

- Do not reuse the supportcushions.
- If less than 45 samples are applied to the miniblotter, fill one neighbouring slot with 2XSSPE/0.1%SDS to prevent cross-flow.
- Use a dedicated plastic container. Do not use this container for other purposes, since some reagents decrease the intensity of the Spoligopatterns.
- If the result is unsatisfactory, you can try to improve this.
  - Black spots (background) possibly occur due to contamination during filter handling (e. g. touched with fingers). Start again from step 8.
  - Blank areas in the spoligopatterns possibly indicate that the membrane was not completely soaked with ECL detection liquid. Start again from step 13.

4. Reuse of the membrane

a) Purpose

The hybridized PCR product is dissociated from the membrane in order to reuse the membrane for the next hybridization. A membrane can be reused for about 15 times.

b) Procedure

1) Wash the membrane twice by incubation in 1% SDS at 80°C for 30 min.
2) Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.
3) Store the membrane at 4°C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

3. Interpretations of results

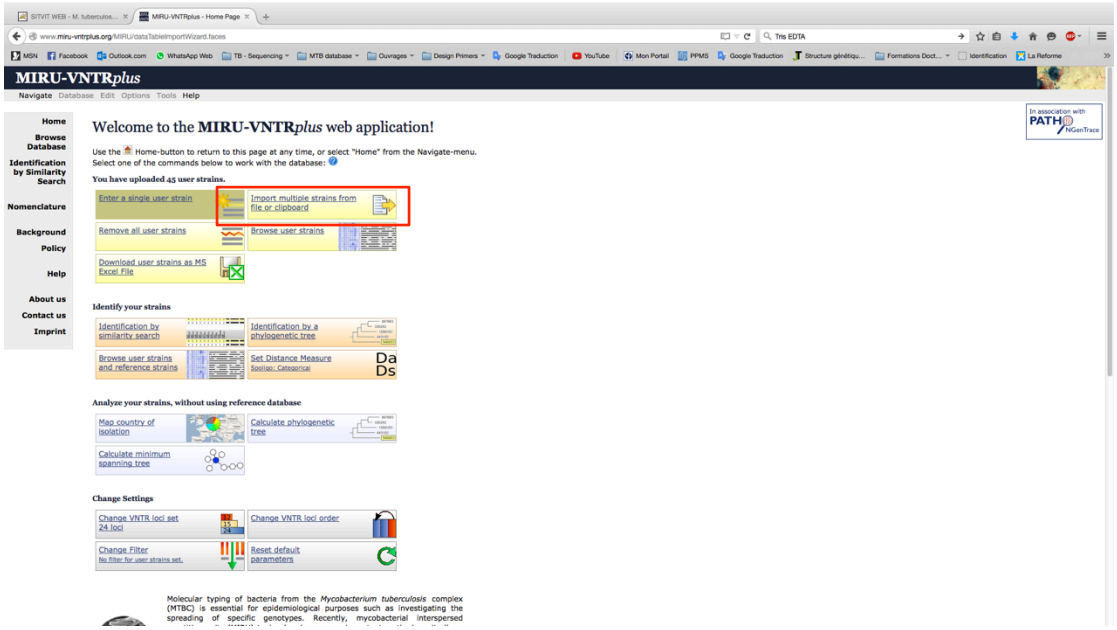
Example 1

Go to http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/description.jsp

y. one by one, copy the spoligotypes (x. step), paste them in the Spoligotype box or download the **excel file** in the **Analyse your data file** menu then fill it, z. click on **Submit** or upload the excel file, aa. The results appear shortly thereafter.

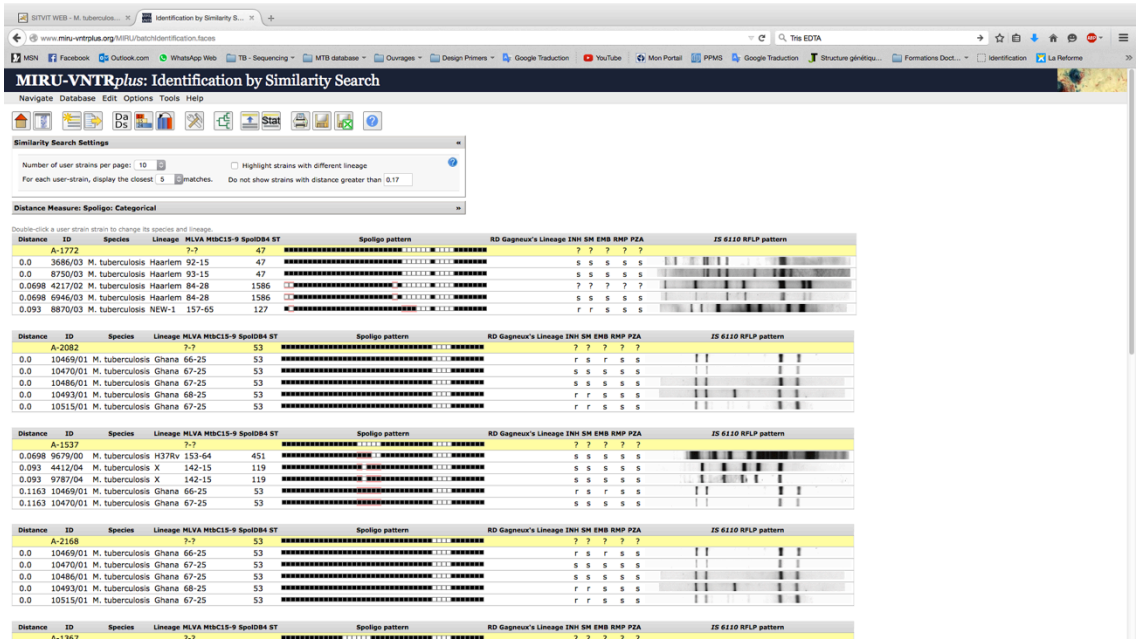
Example 2

Go to: http://www.miru-vntrplus.org/MIRU/index.faces



bb. Import multiple strains from file or clipboard, in the next Internet page; untick MIRU-VNTR, Regions of Difference, Single Nucleotide Polymorphism, and Susceptibility. Click on Next and click on Download Template MS Excel File (.xls).

Copy the values obtained at step x. (or from MIRU-VNTR results), paste them in the downloaded file while taking care of replacing the values n by 1 and o by 0 (zero).



Submit the filed file and you obtain these results. In yellow your strains analyzed and in white, the strain (specie, lineage and SIT) from database corresponding to your submitted spoligotype.

4. Troubleshooting

1) No hybridization signal detected: analyse 5 µl of the PCR product on a 2% agarose gel. A ladder pattern should be visible. If a ladder pattern is visible, check the labelling of the PCR product by spotting it onto a membrane, followed by incubation with streptavidin peroxidase.
2) High background (stripes): thoroughly clean the miniblotter using a dedicated brush, and soak the apparatus, preferably overnight, in a soap solution, e.g. Extran (Merck).
3) High background (spots): strip the membrane again, and test it with PCR products of the control strains. If stripping does not lead to a lower background, the membrane should not be used anymore.

5. Bibliographic references

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SUPPLEMENT 1

Concentration of the aminolink-oligo's.

Oligo number	Oligo name	Concentration (pmol/150 µl)	Oligo number	Oligo name	Concentration (pmol/150 µl)
1	SP-F310A	12.5	**23**	SP-F510A	50.0
2	SP-F320A	30.0	**24**	SP-F520A	50.0
3	SP-F330A	12.5	**25**	SP-F530A	25.0
4	SP-F340A	12.5	**26**	SP-F540A	12.5
5	SP-F350A	12.5	**27**	SP-F550A	25.0
6	SP-F360A	12.5	**28**	SP-F560A	12.5
7	SP-F370A	25.0	**29**	SP-F570A	12.5
8	SP-F380A	50.0	**30**	SP-F580A	12.5
9	SP-F390A	12.5	**31**	SP-F590A	12.5
10	SP-F400A	15.0	**32**	SP-F600A	25.0
11	SP-F410A	30.0	**33**	SP-F610A	100
12	SP-F420A	60.0	**34**	SP-F620A	25.0
13	SP-F430A	12.5	**35**	SP-F630A	12.5
14	SP-F440A	30.0	**36**	SP-F640A	12.5
15	SP-F450A	30.0	**37**	SP-F650A	12.5
16	SP-F455A	12.5	**38**	SP-F660A	25.0
17	SP-F457A	100	**39**	SP-F670A	25.0
18	SP-F460A	12.5	**40**	SP-F680A	12.5
19	SP-F470A	12.5	**41**	SP-F690A	12.5
20	SP-F480A	12.5	**42**	SP-F700A	25.0
21	SP-F490A	25.0	**43**	SP-F710A	50.0
22	SP-F500A	12.5			

SUPPLEMENT 2

Spacer sequences

The sequences of the spacer-specific oligonucleotides are:

1	ATAGAGGGTCGCCGGTTCTGGATCA
2	CCTCATAATTGGGCGACAGCTTTTG
3	CCGTGCTTCCAGTGATCGCCTTCTA
4	ACGTCATACGCCGACCAATCATCAG
5	TTTTCTGACCACTTGTGCGGGATTA
6	CGTCGTCATTTCCGGCTTCAATTTC
7	GAGGAGAGCGAGTACTCGGGGCTGC
8	CGTGAAACCGCCCCCAGCCTCGCCG
9	ACTCGGAATCCCATGTGCTGACAGC
10	TCGACACCCGCTCTAGTTGACTTCC
11	GTGAGCAACGGCGGCGGCAACCTGG
12	ATATCTGCTGCCCGCCCGGGGAGAT
13	GACCATCATTGCCATTCCCTCTCCC
14	GGTGTGATGCGGATGGTCGGCTCGG
15	CTTGAATAACGCGCAGTGAATTTCG
16	CGAGTTCCCGTCAGCGTCGTAAATC
17	GCGCCGGCCCGCGCGGATGACTCCG
18	CATGGACCCGGGCGAGCTGCAGATG
19	TAACTGGCTTGGCGCTGATCCTGGT
20	TTGACCTCGCCAGGAGAGAAGATCA
21	TCGATGTCGATGTCCCAATCGTCGA
22	ACCGCAGACGGCACGATTGAGACAA
23	AGCATCGCTGATGCGGTCCAGCTCG
24	CCGCCTGCTGGGTGAGACGTGCTCG
25	GATCAGCGACCACCGCACCCTGTCA
26	CTTCAGCACCACCATCATCCGGCGC
27	GGATTCGTGATCTCTTCCCGCGGAT
28	TGCCCCGGCGTTTAGCGATCACAAC
29	AAATACAGGCTCCACGACACGACCA
30	GGTTGCCCCGCGCCCTTTTCCAGCC
31	TCAGACAGGTTCGCGTCGATCAAGT
32	GACCAAATAGGTATCGGCGTGTTCA
33	GACATGACGGCGGTGCCGCACTTGA
34	AAGTCACCTCGCCCACACCGTCGAA
35	TCCGTACGCTCGAAACGCTTCCAAC
36	CGAAATCCAGCACCACATCCGCAGC
37	CGCGAACTCGTCCACAGTCCCCCTT
38	CGTGGATGGCGGATGCGTTGTGCGC
39	GACGATGGCCAGTAAATCGGCGTGG
40	CGCCATCTGTGCCTCATACAGGTCC
41	GGAGCTTTCCGGCTTCTATCAGGTA
42	ATGGTGGGACATGGACGAGCGCGAC
43	CGCAGAATCGCACCGGGTGCGG

SUPPLEMENT 3

Preparation of the membrane containing the spacer-oligonucleotides

The spacer- oligonucleotide sequences are derived from DNA sequences of the DR region in the strains *M. tuberculosis* H37Rv and *M. bovis* BCG P3. All spacer-oligonucleotides are synthesized with a 5' terminal aminogroup, by which they can be covalently linked to an activated negatively charged Biodyne C membrane.

1) Dilute the spacer oligonucleotides to the optimized concentrations (see supplement 1) in 150 µl 500 mM $NaHCO_3$, pH 8.4.[a)]

2) Activate the Biodyne C membrane by 10 min incubation in 10 ml freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in demineralized water, in a rolling bottle at room temperature.

3) Place the membrane in a plastic container and shake with demineralised water for 2 min and place it on a support cushion in a clean miniblotter system. Turn the screws hand-tight.

4) Fill the slots of the miniblotter with 150 µl of the diluted oligonucleotide solutions but do not use the first and the last slot to apply oligo's.

5) Use the first and the last slot to mark the edges of the membrane by adding drawing pen ink, diluted 1:100 in 2xSSPE buffer.

6) When all samples are added, incubate for 2 min at room temperature.

7) Remove the oligonucleotide solutions by aspiration in the same order as in which they were applied to the slots.

8) Remove the membrane from the miniblotter using forceps and incubate it in 250 ml freshly prepared 100 mM NaOH for 10 min (maximum) in a plastic container, while shaking, to inactivate the membrane.[b)]

9) Rinse the membrane with demineralised water.

10) Wash the membrane in a plastic container by gentle shaking in 250 ml 2 x SSPE/0.1% SDS for 5 min at 60°C.

11) Wash the membrane in a plastic container by gentle shaking in 100 ml 20 mM EDTA pH 8 for 15 min at room temperature.

12) Store the membrane at 4°C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

Remarks

- The pH of the $NaHCO_3$ should be **exactly** 8.4.
- Inactivation of the membrane by NaOH longer than 10 min will result in weak hybridization signals.

SUPPLEMENT 4

Solutions

500 mM $NaHCO_3$, pH8.4

10.5 g NaHCO3 / 250 ml demineralised water.

16% (w/v) EDAC

1.6 g EDAC / 10 ml demineralised water.

100 mM NaOH

0.8 g NaOH / 200 ml demineralised water.

0.5 M EDTA, pH 8.0

186.12 g EDTA / L demineralised water.

20 mM EDTA, pH 8.0

Dilute 0.5 M EDTA 25 times.

20xSSPE

0.2 M $Na_2HPO_4*2H_2O$	35.6 g/l
3.6 M NaCl	210.24 g/l
20 mM EDTA	7.4 g/l

The pH should be 7.4. Autoclave.

Store at room temperature for no longer than one year.

2xSSPE

Dilute 20xSSPE ten times with demineralised water.

10% SDS

10 g SDS / 100 ml demineralised water.

2xSSPE/0.1%SDS

Add 100 ml 20xSSPE and 10 ml 10% SDS to 890 ml demineralised water.

2xSSPE/0.5%SDS

Add 100 ml 20xSSPE and 50 ml 10% SDS to 850 ml demineralised water.

SUPPLEMENT 5

Supplies

Reagent/Material	Supplier	Order number
Miniblotter MN45	Immunetics, 380 Green street, Cambridge, Mass. 02139, USA. Tel. 617-492-5416, Fax. 617-868-7879, Toll-free: 800-227-4765	MN45
Foam cushions		PC200
"Spacers hybridized" Membrane	**Ocimum Biosolutions Ltd** Royal Demeure, Plot No. 12/2, Sector-1, HUDA Techno Enclave, Madhapur, Hyberabad – 500 081. A.P. India Email: hyb@ocimumbio.com Office: +91-40-6698-6700 Fax: +91-40-6662-7205 **Ocimum Biosolutions LLC**. 134 Vintage Park Blvd., Houston, TX 77070, USA Email: info@ocimumbio.com Phone: (281) 606 4633 Fax: (281) 606 4634	
Biodyne C membrane	Pall Biosupport	BNBCH5R
EDAC (100 g)	SIGMA	E.7750
20 x SSPE, 4 ltr.	Gibco BRL Life Technologies Inc.	15591-035
SDS specially pure (500 g)	BDH Laboratory Supplies	44244 4H
DNA polymerase	EUROBIO	GAETAQ00-4F
Streptavidin-POD-conjugate	Roche	11089153001
ECL detection liquid	Amersham International	RPN2106