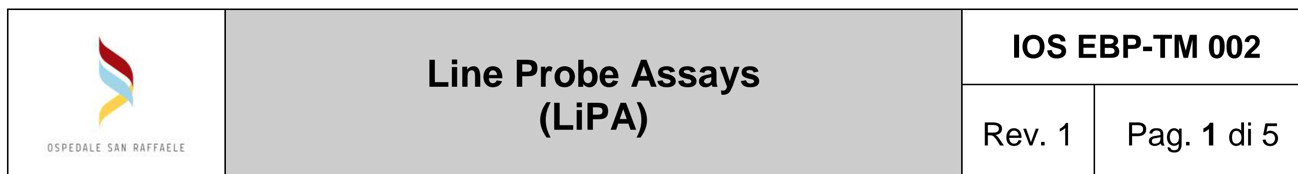

	Line Probe Assays (LiPA)	IOS EBP-TM 002	
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Destinatari: **Coordinatore, Tecnici e Studenti del Settore Genotipizzazione Micobatteri - EBP**

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Rev.	Descrizione modifiche	Data
0	Prima Emissione	31/10/2010
1	Inserimento protocolli GT MTBDRPlus Ver 2.0 e cambio ragione sociale	06/08/2012

Compilazione	Sviluppo	Verifica	Approvazione
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1. SCOPE

This instruction describes the principles and the procedures necessary to perform the Line Probe Assays (LiPA) for the detection, identification of species, identification of mutations involved in the resistant to antitubercle drugs.

2. APPLICATION

LiPA assays are the new frontier of the rapid diagnosis of tuberculosis. This procedure is applicable within the DMA and the TM (advanced diagnostics of mycobacteria) areas. LiPA is a generic definition of a particular type of assays which deploys the reverse hybridization methodology. This panel includes different types of assays.

3. DEFINITIONS AND ABBREVIATIONS

LiPA	Line Probe Assay
MDR	Multidrug resistance
DMA	Diagnosi Microbiologica Avanzata

4. RESPONSIBILITIES

Supervision and the correct application of the following instruction is the responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator. Results should be validated by the area coordinator.

5. EQUIPMENT AND MATERIALS

5.1 Commercial Kits

- GenoType® MTBDR*plus* *Ver 2.0* (Hain Lifescience)
- GenoType® Mycobacterium CM (Hain Lifescience)
- GenoType® Mycobacterium AS (Hain Lifescience)
- GenoType® MTBDR*sl* (Hain Lifescience)
- GenoType® MTBC (Hain Lifescience)

5.2 Kit Contents

Membrane strips coated with specific probes (STRIPS)	12	96
Primers Nucleotide Mix (PNM), contains primers, nucleotides, < 1% Dimethyl Sulfoxide, dye; Master Mix A; Master Mix B	0.5 ml	4 ml
Denaturation Solution (DEN) contains < 2% NaOH, dye *(ready to use)*	0.3 ml	2.4 ml
Hybridization Buffer (HYB) contains 8-10% anionic tenside, dye *(ready to use)*	30 ml	120 ml
Stringent Wash Solution (STR) *(ready to use)* contains > 25% of a quaternary ammonium compound, < 1% anionic tenside, dye	20 ml	120 ml
Rinse Solution (RIN) *(ready to use)* contains buffer, < 1% NaCl, < 1% anionic tenside	50 ml	360 ml
Conjugate Concentrate (CON-C) contains streptavidin-conjugated alkaline phosphatase, dye	0.2 ml	1.2 ml
Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, < 1% NaCl	20 ml	120 ml
Substrate Concentrate (SUB-C) contains Dimethyl Sulfoxide, Substrate solution	0.2 ml	1.2 ml
Substrate Buffer (SUB-D) contains buffer, < 1% MgCl, < 1% NaCl	20 ml	120 ml
Tray, evaluation sheet	2	4
Template	1	4

5.3 Equipment Required

- Absorbent paper
- Adjustable pipettes for 10, 20,200,1000 ul
- Disposable gloves
- Sterile pipettes tips with filter
- PCR tubes
- Shaking platform (Twin Incubator)
- Thermal cycler
- GTBlot 20 for automated procedure

6. PROCEDURES

6.1 Principle of the Procedure

The Genotype® assay platform is based on DNA•STRIP® technology and permits the molecular identification of mycobacterial species including the most common Non tubercle Mycobacteria (NTM) and *Mycobacterium tuberculosis complex* as well as the discrimination at species level within the *M.tb complex*. The Genotype® MTBDR assays allows the detection of the most common mutations involved in drug resistances to: Rifampicin, Isoniazide, Ethambutol, Fluoroquinolones, Aminoglycosides, Capreomycin and Viomycin.
The methodology is DNA-based which requires DNA isolation as the first step of the procedure. DNA can be isolated from positive cultures or directly from biological samples (respiratory or non-respiratory specimen) confirmed microscopy positive (SS+). The whole procedure is divided into three steps: DNA extraction (from positive cultures or clinical specimen decontaminated by NALC-NaOH; a multiplex PCR amplification with biotinylated primers and reverse hybridization. The hybridization includes a chemical denaturation of the amplification products, hybridization of a single-stranded, biotin-labeled amplicons to the membrane-bound, stringent washing, addition of a streptavidin/alkaline phosphatase (AP)conjugate and a AP mediated staining reaction.

6.2 PCR Amplification Protocols

PCR Mixes are prepared according to manufacturer's instructions.

Reagents	Vol. (µl)	Assays	Reagents	Vol. (µl)	Assays
PNM	35.0	GT sl; GT Mycobacterium CM and AS; GT MTBC	Mix B	35.0	GT MTBDRPlus Ver 2.0
Buffer 10x	5.0		Mix A	10.0	
$MgCl_2$	2.0		DNA solution	5.0 /each	
Sterile water	3.0				
Taq Polymerase	0.2/0.4				
DNA solution	5.0 /each				

Amplification cycles are indicated in the following table.

GT MTBDRplus ver 1; CM/AS; MTBC; GT MTBDRsl				GT MTBDRplus ver 2.0			
Time	T °C	Culture	Clinical Specimen	Time	T °C	Culture	Clinical Specimen
15 min	95°	1 cycle	1 cycle	15 min	95°	1 cycle	1 cycle
30 sec	95°	10 cycles	10 cycles	30 sec	95°	10 cycles	20 cycles
2 min	58°			2 min	65°		
25 sec	95°	20 cycles	40 cycles	25 sec	95°	20 cycles	30 cycles
40 sec	53°			40 sec	50°		
40 sec	70°			40 sec	70°		
8 min	70°	1 cycle	1 cycle	8 min	70°	1 cycle	1 cycle
Infinito	6°			Infinito	6°		

6.3 Hybridization

The procedure of hybridization and development of the assays is the same for every assay.
The following steps are for the development of the assays manually:

Pre-warm the Twin Incubator to 45 ℃ and HYB (green) and STR (red) solutions to 37- 45 ℃ in a water bath.
Leave the other reagents (besides CON-C and SUB-C that must be stored at 2-8℃ till their use) equilibrate at room temperature (around 30 minutes)
Take with forceps the number of strips necessary for the running assay and mark them on the left side of the blue/red/green line with the number corresponding to the sample
Take 1 or 2 strips' trays.
Dispense 20 µl of DEN solution on the bottom corner of each lane of the tray.
Add to each DEN solution 20 µl of PCR product, pipetting up and down a couple of times to mix well. Let it stand 5 minutes at room temperature.
Add to each lane 1 ml of pre-warmed HYB solution. Shake gently until the solution appears coloured evenly.
Place the corresponding strip in the respective lane. Be sure the strip is well covered by the HYB solution.
Place the tray into the Twin Incubator and turn on the shaking mode (frequency 300-350 rpm). Close the lid and let it incubate 30 min at 45℃ while sh aking.
Completely aspirate the hybridization solution
Add 1 ml of STR solution (red) to each lane and incubate 15 minutes at 45℃ while shaking (300-350 rpm)
In the meantime, prepare the Conjugate Working solution and the Substrate Working solution:
_ The two working solutions are prepared by making a 1:100 solution of the concentrated reagents (CON-C and SUB-C) with the respective diluted reagents (CON-D and SUB-D). Make a calculation provided that for each sample 1 ml of Conjugate and Substrate working solution is required.
Remove completely the STR solution by pouring off the liquid into a container and turn the tray upside down on an absorbent paper.
Wash each strip with 1 ml of RIN solution for 1 minute at room temperature. Shake the tray gently.
Remove completely the RIN solution by pouring off the liquid into a container and turn the tray upside down on an absorbent paper.
Add 1 ml of Conjugate solution to each strip and incubate 30 minutes at room temperature while shaking (use the Twin Incubator set previously at 25 ℃)
Remove completely the Conjugate solution by pouring off the liquid into a container and turn the tray upside down on an absorbent paper.
Wash each strip twice with 1 ml RIN solution for 1 minute. Shake gently. Remove completely the solution at the end of each step.
Wash each strip once with 1 ml distilled water. Shake gently. Remove water.
Add 1 ml of Substrate solution to each strip and incubate around 6 minutes at room temperature. Cover the trays with an aluminium foil. In case of bands not well developed it is possible to extend the incubation time up to 25 minutes.
Stop the reaction by adding water and pour off the solutions.
Using forceps, place each strip onto absorbent paper for letting them dry.

6.3.1 Hybridization using the GT- Blot 20

By using the GT-Blot 20 it is possible to test 20 strips at a time. It is an automated procedure to develop Hain Assays. Volumes, incubation times and temperatures are the same as those described in the Paragraph 6.3, but few adjustments in the procedure have to be taken into account at the beginning of the methodology:
Prepare all the solutions necessary for the assay (included Conjugate and Substrate working solutions), before processing PCR products. Try to estimate volumes taking a "plus three samples" in account.
Pour the solutions into specific flasks, indicated with different coloured flags (*see GTBlot Manual).* Follow the instruction appearing on the small display in order to fill all pumps (6 in total) to be ready for the assay. It will be activated the "Pre-warming system" that lasts 30 minutes.

In the meantime mark all the strips necessary for a specific run (up to 20) and let them lay out of the tray.
Take the GT-Blot 20 specific tray. Dispense 20 µl of DEN solution on the bottom corner of each lane of the tray.
Add to each DEN solution 20 µl of PCR product, pipetting up and down a couple of times to mix well. Let it stand 5 minutes at room temperature.
Place the corresponding strip in the respective lane avoiding touching the mixed DEN-DNA solution. Place it on the top side of the lane.
Take the tray and place it into the GT-Blot 20. Start the programme named "GT-45"
At the end of the programme, take the strips out and let them dry onto absorbent paper.

7. RECORDING AND REPORTING

Once the strips are developed and dried, stick them on the Worksheet included into the commercial kits. They could be attached by using glue and adhesive tape. Each strip must be interpreted according to the manuals. For Genotype MTBDR*plus* and Genotype MTBDR*sl*, spaces available in the interpretation table on the worksheet have to be filled as follow:

TUB	rpoB-wt	rpoB-MUT	katG-wt	katG-MUT	inhA-wt	inhA-MUT		RIF		INH	
								SENS	RES	SENS	RES
+	8	3	-	-	1	1			R		R
+	-	-	-	-	-	-		S		S	

How to read the table:
- TUB + : M. tb complex presents
- rpoB wt; katG wt; inhAwt, gyrA wt… : indicate the wild-types bands that are missing
- rpoB MUT; katG MUT, inhA MUT…: indicate the mutation that is present

Worksheets and results have to be attached to the laboratory register "LiPA" indicating the date of execution of the test and the name of the operator.

8. RELATED DOCUMENTS

- GenoType® MTBDR*plus* Ver 2.0 (Hain Lifescience) Package Insert
- GenoType® Mycobacterium CM (Hain Lifescience) Package Insert
- GenoType® Mycobacterium AS (Hain Lifescience) Package Insert
- GenoType® MTBDR*sl* (Hain Lifescience) Package Insert
- GenoType® MTBC (Hain Lifescience) Package Insert