Video Article

Demonstrating a Multi-drug Resistant *Mycobacterium tuberculosis* Amplification Microarray

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URL: <http://www.jove.com/video/51256>

DOI: [doi:10.3791/51256](https://doi.org/10.3791/51256)

Keywords: Immunology, Issue 86, MDR-TB, gel element microarray, closed amplicon, drug resistance, rifampin, isoniazid, streptomycin, ethambutol

Date Published: 4/25/2014

Citation: Linger, Y., Kukhtin, A., Golova, J., Perov, A., Qu, P., Knickerbocker, C., Cooney, C.G., Chandler, D.P. Demonstrating a Multi-drug Resistant *Mycobacterium tuberculosis* Amplification Microarray. *J. Vis. Exp.* (86), e51256, doi:10.3791/51256 (2014).

Abstract

Simplifying microarray workflow is a necessary first step for creating MDR-TB microarray-based diagnostics that can be routinely used in lower-resource environments. An amplification microarray combines asymmetric PCR amplification, target size selection, target labeling, and microarray hybridization within a single solution and into a single microfluidic chamber. A batch processing method is demonstrated with a 9-plex asymmetric master mix and low-density gel element microarray for genotyping multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB). The protocol described here can be completed in 6 hr and provide correct genotyping with at least 1,000 cell equivalents of genomic DNA. Incorporating on-chip wash steps is feasible, which will result in an entirely closed amplicon method and system. The extent of multiplexing with an amplification microarray is ultimately constrained by the number of primer pairs that can be combined into a single master mix and still achieve desired sensitivity and specificity performance metrics, rather than the number of probes that are immobilized on the array. Likewise, the total analysis time can be shortened or lengthened depending on the specific intended use, research question, and desired limits of detection. Nevertheless, the general approach significantly streamlines microarray workflow for the end user by reducing the number of manually intensive and time-consuming processing steps, and provides a simplified biochemical and microfluidic path for translating microarray-based diagnostics into routine clinical practice.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51256/>

Introduction

Early case detection and rapid treatment are considered the most effective control strategies to reduce *Mycobacterium tuberculosis* (MTB) transmission¹, and there is now a broad consensus in the TB community that a point of care (POC) or near POC test to simultaneously diagnose TB and drug resistance (DR) is needed. Technologies such as Cepheid's GeneXpert and other nucleic acid amplification tests reduce the time to diagnosis for many TB patients, and provide a rapid read-out indicating resistance to rifampin or selected mutations conferring resistance to other first or second line drugs². Although real-time and isothermal nucleic acid amplification tests are designed to identify the drug resistance mutations that lead to MDR-TB, the spectrum of mutations they detect is often inadequate to design an individualized drug regimen corresponding to the drug resistance profile of the patient, and technical constraints related to optical cross-talk or the complexity of amplification and reporting chemistries³⁻⁷ may limit the number of loci or mutations that are detected. Thus, detection technologies with higher multiplexing capacity are required to address known gaps in MDR-TB POC diagnostics.

Microarrays and the WHO-endorsed Hain line probe assays can address the "multiple gene, multiple mutations" challenge of diagnosing MDR-TB⁸⁻²⁹. Unfortunately, these hybridization-based, multiplexed detection platforms use multistep, complicated, and open-amplicon protocols that require significant training and proficiency in molecular techniques. The amplification microarray³⁰ was designed to address some of these microarray work-flow and operational concerns. The simplifying fluidic principles are to amplify, hybridize, and detect nucleic acid targets within a single microfluidic chamber. The user introduces the nucleic acid and amplification master mix into a fluidic chamber with a pipette and starts the thermal cycling protocol. For the batch processing method shown here, microarrays are subsequently washed in bulk solution, dried, and imaged. This study demonstrates the functionality of an amplification microarray using an MDR-TB microarray test for *rpoB* (30 mutations), *katG* (2 mutations), *inhA* (4 mutations), *rpsL* (2 mutations), *embB* (1 mutation), IS1245, IS6110, and an internal amplification and hybridization control. At least one matched pair of microarray probes (wildtype (WT) and single-nucleotide mutant (MU)) is included for each mutation of interest. Purified nucleic acids from multi-drug resistant *M. tuberculosis* are from the TDR Tuberculosis Strain Bank³¹. Gel element microarrays are manufactured on glass substrates by copolymerization essentially as described elsewhere³², except that we use 4% monomer and 0.05 mM each probe in the polymerization mixture. Arrays are surrounded with a 50 ml gasket prior to use. After thermal cycling, hybridization, and wash steps, amplification microarrays are imaged on an Akonni portable analyzer. Background-corrected, integrated signal intensities are obtained from the raw .tif images using a fixed circle algorithm. Noise for each gel element is calculated as three times the standard deviation of the local spot backgrounds. Gene targets are typically considered detectable for signal to noise ratio (SNR) values ≥ 3 . In order to determine the

presence or absence of a specific mutation in each gene or codon, a discriminant ratio is calculated from the SNR values as (WT-MU)/(WT+MU). Discriminant ratios <0 are indicative of a drug-resistance mutation at the locus, whereas ratios >0 are indicative of the wild-type sequence.

Protocol

For laboratories that follow universal PCR precautions, it is operationally more efficient to include several amplification microarrays and gaskets per substrate and wash all amplification microarrays simultaneously in a bulk container, as described here. Consumable formats are available for performing post-amplification microarray washing steps in an entirely sealed, closed amplicon test, as reported elsewhere^{30,33}.

1. Setup

1. Extract and purify nucleic acids from the sample under appropriate biosafety conditions with a method of choice. The requirement is that nucleic acids be of sufficient purity for asymmetric, multiplex PCR amplification.
2. Prepare workspace by wiping surfaces and equipment with decontaminating solutions.
3. Place amplification reagents on ice or cold block.
4. Label a sterile 1.5 ml microfuge tube for the amplification master mix, and label a sterile 0.2 ml microfuge tube for each sample.
5. After reagents are thawed, briefly vortex and collect contents to the bottom of the tube with a pulse-spin in a mini-centrifuge. Do not vortex *Taq* polymerase. Gently flick the tube to mix and follow with a pulse-spin in mini-centrifuge.
6. Prepare an amplification master mix using the per-sample reaction volumes shown in **Table 1**. To account for possible pipetting inaccuracies, prepare at least one more reaction volume than the total number of samples being processed. Note that the master mix contains additional *Taq* polymerase, over and above what is provided with the multiplex PCR buffer. Only add the amplification/inhibition control to the master mix after all other reagents are combined, and the stock reagent tubes returned to storage.

Reagent	Per- Sample Volume (μl)	Final []
Multiplex PCR Buffer with HotStar <i>Taq</i> Plus	25	1x
Bovine serum albumin (BSA)	0.55	0.6 mg/ml
Formamide	3.8	7.6%
Additional <i>Taq</i> polymerase	0.8	units/μl (4 units total)
MDR-TB primer mix	15.75	-
RNase-free H ₂ O	2.1	-
Amplification/Inhibition Control	1	5.0 fg/μl
Total	49	

Table 1. MDR-TB amplification microarray master mix composition.

7. Vortex the master mix and collect contents to the bottom of the tube with a pulse-spin in a mini-centrifuge.
8. Combine 49 μl of master mix and 1 μl of *M. tuberculosis* DNA to each of the sample tubes from step 1.4, above. For an external, no template control (NTC), use 1 μl of molecular biology grade water in place of the *M. tuberculosis* DNA sample. Vortex and pulse-spin the tube(s) when finished.

2. Load Amplification Microarrays

1. Add 48 μl of each master mix/sample onto the center of their respective microarrays, being careful to not touch the microarray itself.
2. Place a cover slip on top of the microarray gasket and seal, being careful not to trap any air bubbles in the microarray chamber. Air bubbles can negatively affect amplification efficiency and may create artifacts or noise in the subsequent microarray image.

3. Thermal Cycling

1. Once all microarrays are loaded with sample, place substrates on the flat block thermal cycler. Make sure that the heated lid option is turned "Off". Equipment obtained from Akonni Biosystems will already be prequalified for use. Otherwise, it is very important to verify that the flat block thermal cycler(s) provide(s) uniform, consistent heating across all microarray substrates.
2. Open the thermal cycler software, select the appropriate program, enter "50 μl" for the reaction volume, and initiate the run. The thermal cycling protocol described here consists of:

Thermal Cycling Steps		
1	88 °C	5 min
2	88 °C	30 sec
3	55 °C	1 min
4	65 °C	30 sec
5	Repeat steps (2-4) for 50 cycles	
6	65 °C	3 min
7	55 °C	3 hr

- The total number of thermal cycles and 55 °C post-amplification hold time are independent variables that the user may modify, as needed or appropriate for the specific experiment. Because the MDR-TB master mix creates asymmetric (predominantly single-stranded) amplicons, it is usually helpful to include more thermal cycles than might otherwise be utilized for a conventional, exponential PCR amplification protocol.
- When the thermal cycling and hybridization program is complete, end the program, remove the amplification microarrays, close the software, and turn off the thermal cyclers(s).

4. Wash and Dry

- For washing up to 24 substrates simultaneously, prepare at least 250 ml 1x SSPE – 0.1% Triton X-100 wash buffer, and two containers with at least 250 ml deionized or Milli-Q grade water each. The wash buffer can be prepared in advance.
- Carefully remove the cover slip and gasket from each amplification microarray chamber using flat-end forceps. **This step is the point at which there is the greatest risk of physically damaging gel element arrays.** Use appropriate PCR contamination controls to prevent the spread of amplified nucleic acids within the work environment.
- Place microarray substrates in a histology slide holder, and place all slides into the wash bin containing the wash buffer. Cover the container with a lid.
- Wash microarrays for 10 min at room temperature with gentle agitation.
- Dip the microarrays three times each in two successive washes of deionized or Milli-Q grade water, and air dry.

5. Imaging

The asymmetric MDR-TB primer mix generates Cy3-labeled amplicons. Gel element microarrays can be imaged with any standard microarray imager capable of imaging Cy3. The following imaging procedure is specific for the MDR-TB master mix, Dx2100 field portable imager, and automated analysis software provided by Akonni.

- Ensure that the ethernet cable from the imager is connected to the computer.
- Turn on the imager. The power switch is located on the back panel of the instrument. Blue LEDs on the front of the imager will illuminate when imager is powered on.
- Turn on the computer.
- On the computer desktop, double click the software icon to launch the automated image analysis software.
- Wait for the software to establish a connection with the imager camera. Once the connection is established, the Analyze button becomes available, and the message "Connection Successful" will appear in the lower left corner of the screen.
- Insert the dried amplification microarray with the microarray facing **away** from the user, and **toward** the objective lens. If the microarray is improperly oriented with respect to the lens and camera, the automated analysis software will fail to place a grid on the fluorescence image.
- Close the access door. A preview image will be available on the computer screen.
- Select the MDR-TB analysis script from the drop-down menu and enter the desired Exposure Time (in milliseconds). A typical starting point for gel element amplification microarrays is 100-500 msec.
- Saturated pixels will be displayed on the Preview Image in red. Adjust the exposure time to minimize the number of saturated pixels within individual spots.
- Click Analyze to acquire and analyze the fluorescence image. The software will automatically place an MDR-TB assay-specific grid onto the image, extract integrated intensity and background values, and report drug resistance and genotyping results. The automated analysis will typically take a few seconds. For challenging images that contain scratches, dust, fluorescent artifacts, or physical damage to the microarray features, the software may need up to 1 min to complete the analysis.
- Once the analysis is complete, click on Save, select the destination folder, type in a filename, and click OK. Underlying microarray data are saved as an .xls file and can be exported for off-line analyses, if desired.
- Once the analysis is complete, remove the amplification microarray from the imager, and click on New to initiate the next analysis.
- When finished collecting data, close the software, shut down the computer, and turn off the imager.

Representative Results

Qualitative image analysis can provide insight into sources of experimental noise or variability that are challenging to identify in data tables generated by automated image analysis software. Thus, it can be useful to visually ascertain that 1) all gel elements are intact and undamaged, 2) the global background is free from fluorescent artifacts that might affect individual signal to noise ratio (SNR) values, 3) there is no evidence for bubble formation or nonuniform amplification/hybridization across the array, and 4) that the software accurately identified all spots on the microarray image. Example MDR-TB amplification microarray images are shown in **Figure 1**, illustrating a high-quality array and artifacts that may arise due to physical damage or bubbles during thermal cycling. Standard microarray image analysis software is usually able to place a

grid and extract integral intensity and background data even from poor quality images such as shown in **Figure 1B**, so it is incumbent upon the researcher to establish quality control criteria and metrics for accepting or rejecting microarray images from further analysis. Probe redundancy may help ameliorate these issues. However, a fully automated, diagnostic MDR-TB amplification microarray reporting algorithm would otherwise declare the test from **Figure 1B** as "invalid".

Amplification microarray SNR values for a dilution series of wildtype H37Ra genomic DNA are shown in **Table 2**. At 6.25 pg input genomic DNA per reaction or approximately 1.25×10^3 cell equivalents, all wildtype probes are readily detected. SNR values for each of the internal amplification and inhibition controls ranged from 98.48 (*rpoB*) to 967.24 (Akonni-supplied internal positive control), indicating that all gene targets in the asymmetric multiplex amplification reaction are amplified and detectable. No template controls were negative (SNR <3) for all probes in the amplification microarray. Genotyping ratios at 6.25 pg input DNA ranged from 0.18-1.00 for all WT/MU probe pairs, which demonstrates correct behavior of WT and MU probes and appropriate genotyping.

Representative genotyping data for a panel of World Health Organization reference isolates of known genotype are shown in **Table 3**. If a single nucleotide polymorphism (SNP) is represented on the gel element microarray, then the amplification microarray test accurately detects the corresponding mutation in the MDR-TB genome. Some of the amplification microarray probes are also sensitive to near-neighbor mutations that are not explicitly represented on the array as a unique probe. For example, isolate TDR-0129 contains an S531E mutation in the *rpoB* gene, but there is not a specific probe on the amplification microarray for S531E. Nevertheless, five other SNP probes targeting codon 531 indicate that a mutation is present at codon 531 — the amplification microarray therefore correctly indicates that this isolate is rifampin resistant. Similar sensitivity to the *rpoB* S513W mutation is evident for isolate TDR-0148. On the other hand, some SNP probes are not sensitive to near neighbor mutations, as illustrated by isolate TDR-0148 and the *rpoB* S512G mutation, and isolate TDR-0011 and the *katG* S315R mutation. We suspect that this type of probe behavior is a consequence of secondary and tertiary structure in the single-stranded amplicon and/or probe³⁴, and is not something that can be predicted *a priori*. Nevertheless, probe sensitivity to near neighbor mutations is analogous to the use of sloppy molecular beacons to detect multiple drug resistance mutations with a minimal set of real-time PCR probes^{35,36}, and can be diagnostically advantageous provided the test does not generate false positives relative to the phenotypic drug susceptibility.

Figure 1. (A) Example amplification microarray image for 100 pg wildtype *M. tuberculosis* H37Ra genomic DNA and a 100 msec exposure time. Cy3 beacons (for automated gridding and segmentation software) are on the periphery of the image. **(B)** Image of an amplification microarray showing a fluorescent halo artifact resulting from bubble formation during thermal cycling, and damaged gel elements/debris. Automated image analysis software is still able to place a grid and extract data from this image. [Please click here to view a larger version of this figure.](#)

Company	Catalog Number
Akonni Biosystems	Inquire
Qiagen	#206143
Qiagen	#201207
Qiagen	#206143
Thermo Fisher Scientific, Inc.	#BP227-500
Sigma-Aldrich	#3B6917
Akonni Biosystems	Inquire
Akonni Biosystems	Inquire
Thermo Fisher Scientific, Inc.	#BP1328-4
Thermo Fisher Scientific, Inc.	#BP151-500
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Decon Labs, Inc.	#8416
Company	Catalog Number
Akonni Biosystems	100-20011
Akonni Biosystems	100-10021
ArrayIt	HTW
Thermo Fisher Scientific, Inc.	#10-300
VWR	#3365040
VWR	#93000-196
Central Pneumatic	#95630

Table 2. Required Materials and Equipment.

	Codon	Probe ID	WT Probe SNR Values					
			500 pg	100 pg	50 pg	25 pg	12.5 pg	6.25 pg
rpoB	507	1	900.22	644.46	523.17	431.08	364.49	287.47
	510	3	1016.37	699.38	600.16	525.07	446.51	361.64
	511	5	867.67	611.97	529.90	451.73	403.76	307.68
	512	8	810.69	544.04	488.85	436.89	391.62	257.46
	513	11	824.36	547.04	496.00	472.01	408.96	242.25
	515	15	715.48	536.59	416.96	335.81	267.48	189.10
	516	17	723.83	496.44	402.95	329.09	270.10	201.98
	522	27	674.37	413.33	360.40	316.75	236.19	153.40
	524	29	269.46	153.69	117.71	118.02	110.13	51.22
	526	31	136.37	109.90	82.63	76.76	53.61	37.76
	531	44	219.92	136.31	109.16	96.18	80.58	26.44
	533	51	130.54	83.60	76.03	63.47	61.35	41.54
rpsL	43	54	10.41	12.75	53.30	767.18	5.39	362.42
	88	56	655.54	542.79	527.43	690.34	403.86	456.05
katG	315	58	1037.03	873.46	816.64	974.83	811.79	876.47
embB	306	66	940.81	781.13	788.75	837.65	787.05	696.69
inhA	8	82	1069.43	934.38	862.58	936.88	809.85	682.56
	15	85	1111.66	931.88	918.22	957.87	795.72	723.16
	17	87	1114.49	926.03	920.60	970.70	854.15	744.41

Table 3. Signal to noise ratios for wildtype probes and a dilution series of *M. tuberculosis* H37Ra genomic DNA. SNR values > 3 are considered detectable.

Isolate ID and genotype			TDR-0005	TDR-0007	TDR-0011	TDR-0012	TDR-0013	TDR-0032	TDR-0086	TDR-0116	TDR-0129	TDR-0148	TDR-0155
			WT	S531L	L533P	M515I D516Y	H526R S503R*	H526Y	D516V	L511P	S531E*	S512G* S531W	S531L
				S315T	S315R*	WT	WT	S315T	S315T	S315T	WT	S315T	S315N
				K43R	K88R			K43R	WT	WT		K43R	WT
				M306I	M306I E378A*			M306I		M306I D354A*		WT	M306I
				WT	WT			WT	C15T	WT			WT
Gene	Mutation	Probe Pair	Discriminant Ratio										
rpoB	S07-DEL	1 2	0.92	0.94	0.96	0.93	0.90	0.95	0.95	0.97	0.95	0.99	0.96
	Q510H	3 4	0.18	0.20	0.20	0.21	0.60	0.23	0.26	0.49	0.23	0.74	0.22
	L511P	5 6	0.74	0.74	0.76	0.76	0.81	0.76	0.78	-0.79	0.78	0.75	0.81
	L511R	5 7	0.85	0.87	0.88	0.85	0.83	0.86	0.84	0.20	0.87	0.81	0.89
	S512T	8 9	0.48	0.47	0.45	0.35	0.72	0.50	0.51	0.36	0.52	0.62	0.52
	S512R	8 10	0.87	0.87	0.89	0.85	0.89	0.89	0.74	0.08	0.89	0.77	0.92
	Q513L	11 12	0.89	0.89	0.95	0.22	0.95	0.93	0.71	0.87	0.92	0.91	0.96
	Q513K	11 13	0.88	0.86	0.90	0.23	0.90	0.90	0.70	0.77	0.90	0.93	0.94
	Q513P	11 14	0.78	0.79	0.77	0.34	0.72	0.79	0.70	0.77	0.82	0.91	0.81
	M515I	15 16	0.84	0.85	0.86	-0.11	0.86	0.88	0.43	0.91	0.87	0.64	0.84
	D516E	17 20	0.90	0.93	0.96	0.17	0.95	0.94	0.66	0.94	0.93	0.95	0.95
	D516Y	17 23	0.12	0.17	0.15	-0.20	0.09	0.18	0.25	0.37	0.16	0.29	-0.01
	D516G	17 24	0.86	0.89	0.90	0.02	0.89	0.88	0.55	0.90	0.88	0.92	0.88
	D516V	17 25	0.73	0.76	0.76	-0.01	0.71	0.76	-0.77	0.85	0.76	0.86	0.70
	S522L	26 28	0.90	0.88	0.88	0.89	0.90	0.86	0.88	0.83	0.90	0.91	0.90
	S522L	27 28	0.91	0.87	0.89	0.91	0.91	0.91	0.89	0.86	0.91	0.92	0.92
	L524S	29 30	0.89	0.92	0.97	0.83	0.77	0.40	0.86	0.88	0.96	0.89	0.97
	H526D	31 32	0.91	0.97	0.96	0.93	0.75	0.46	0.91	1.00	0.96	0.97	0.91
	H526R	31 34	0.78	0.90	0.94	0.66	-0.77	0.08	0.92	0.81	0.91	0.88	0.90
	H526L	31 35	0.71	0.90	0.88	0.63	0.17	0.20	0.88	0.86	0.85	0.89	0.81
	H526Q2	31 36	0.89	0.96	0.96	0.92	0.35	0.73	1.00	0.94	0.96	0.96	0.94
	H526Q1	31 37	0.86	0.97	0.97	0.80	0.70	0.41	1.00	0.87	0.97	0.95	0.95
	H526C	31 38	0.93	0.98	0.97	0.93	0.59	0.30	1.00	0.94	0.98	0.97	0.96
	H526N	31 39	0.83	0.94	0.96	0.88	0.64	0.26	0.90	1.00	0.95	0.97	0.95
	H526P	31 41	0.70	0.89	0.84	0.72	0.34	0.50	0.52	0.84	0.77	0.88	0.79
	H526Y(1)	31 42	0.92	0.98	0.97	0.92	1.00	-0.39	1.00	0.93	0.97	0.98	1.00
	H526Y(2)	31 43	0.96	0.82	0.72	0.63	0.21	-0.91	0.75	0.73	0.58	0.66	0.63
	S531L	44 45	0.92	-0.59	0.03	0.86	0.96	0.91	0.81	0.86	-0.47	0.07	-0.69
	S531L(2)	44 47	0.83	-0.92	0.39	0.86	0.89	0.85	0.71	0.77	-0.64	-0.62	-0.75
	S531W	44 46	0.81	0.19	0.13	0.85	0.90	0.88	0.72	0.88	-0.32	-0.93	0.70
	S531Q	44 48	0.97	0.77	0.75	0.97	0.98	0.97	1.00	1.00	0.28	-0.03	0.87
	S531C(1)	44 49	0.81	0.03	-0.12	0.83	0.84	0.88	0.57	0.68	-0.54	-0.64	0.51
	S531C(2)	44 50	0.79	-0.26	0.04	0.81	0.83	0.87	0.57	0.81	-0.60	-0.88	0.31
	L533P(1)	51 52	0.90	0.60	-0.92	0.88	0.88	0.77	0.91	0.76	0.78	0.80	0.61
	L533P(2)	51 53	0.94	0.77	-0.69	0.94	1.00	0.81	1.00	1.00	1.00	1.00	0.45
rpsL	K43R	54 55	0.22	0.55	0.88	0.19	0.93	-0.37	0.27	0.93	0.24	-0.76	0.18
	K88R	56 57	0.89	-0.50	-0.55	0.89	0.90	0.89	0.90	0.89	0.90	0.90	0.92
katG	S315T	59 62	0.89	-0.87	0.44	0.91	0.90	-0.72	0.66	-0.72	0.90	-0.70	0.35
	S315N	60 79	0.87	0.71	0.66	0.89	0.86	0.41	0.96	0.53	0.89	0.32	-0.68
embB	M306I	66 68	0.74	-0.95	-0.95	0.73	0.69	-0.96	0.74	-0.24	0.72	0.76	-0.51
inhA	8A	82 83	0.20	0.18	0.25	0.31	0.19	0.38	0.85	0.55	0.22	0.38	0.33
	8C	82 84	0.09	0.10	0.15	0.18	0.08	0.17	0.73	0.43	0.11	0.19	0.14
	C15T	85 86	0.59	0.55	0.56	0.66	0.51	0.67	-0.15	0.84	0.61	0.71	0.68
	17T	87 88	0.16	0.26	0.21	0.25	0.13	0.32	0.85	0.44	0.15	0.30	0.28

Table 4. Representative genotyping data from MDR-TB isolates of known genotype at 25 pg per amplification microarray reaction. Asterisks identify mutations that are not represented by a unique probe on the gel element microarray. WT = wildtype nucleic acid sequence. Negative values (bold and shaded) are indicative of a mutation, hence phenotypic drug-resistance. [Please click here to view a larger version of this figure.](#)

Discussion

The extent of multiplexing with an amplification microarray is ultimately dictated by the efficiency of multiplex asymmetric PCR, not the microarray. In our experience, 10-12 unique primer pairs can be readily multiplexed in an amplification microarray format. Conventional primer and probe design criteria therefore apply to new assays, except that one also needs to consider potential interactions between solution-phase nucleic acids and immobilized microarray probes, the thermal efficiency of the thermal cycler, and probe hybridization behavior in a PCR buffer that also contains PCR primers and low molecular weight amplification artifacts. Highly multiplexed amplification approaches such as whole genome amplification are not conducive to a single-chamber amplification microarray protocol for a number of technical reasons, including interference between random hexamers and immobilized probes, and inefficient hybridization of long, double-stranded amplicons. There is also an inter-relationship between ease-of-use, total analysis time, and limits of detection that individual users will need to consider when designing custom assays or using the MDR-TB test described here. For example, reducing the number of amplification cycles and even eliminating the post-amplification hybridization step will significantly reduce total analysis time, but at the expense of test sensitivity. On the other hand, achieving a reproducible 10 gene copy detection limit may require more amplification cycles or hybridization time, thus extending the total analysis time beyond a single work shift.

The genes, mutations, imager, analysis software, and reporting algorithm described here are illustrative of the amplification microarray method and system, rather than a report on their analytical performance and clinical utility. For example, the starting sample can be sputum, decontaminated sediment, solid- or liquid cultures — the requirement for the amplification microarray is simply that the extracted nucleic acids are amplifiable by asymmetric, multiplexed PCR. Some researchers or clinicians may find little to no clinical value in detecting rpsL or embB mutations that confer resistance to streptomycin and ethambutol, respectively, only resistance to rifampin and isoniazid define MDR-TB. Thus, these PCR primers and microarray probes can be replaced with primers and probes that target genes and mutations conferring resistance to other first- or second-line antibiotics. It is possible to write a reporting algorithm to provide the user with detailed information on the specific mutations that are detected in a given specimen, rather than a simple “resistance detected” or “resistance not detected” result. For those

researchers interested in using this specific assay to analyze *M. tuberculosis* isolates, it is also possible to provide the genotyping data even if the IS6110 element is not detected in the specimen.

Regardless of the possible assay and reporting permutations, the amplification microarray and protocol described here is designed to significantly simplify microarray workflow by combining multiple molecular biology steps into a single biochemical reaction and microfluidic chamber. The representative data demonstrate the efficacy of approach by amplifying nine MDR-TB genomic regions and detecting those asymmetric amplicons with a low-density gel element microarray. The protocol described here uses a bulk washing procedure that requires the user to physically remove the gasket and cover slip from the amplification microarray before washing, and is therefore more appropriate for research-use-only applications rather than clinical diagnostics. As described elsewhere, however, it is certainly possible to incorporate a washing step on-chip using lateral-flow fluidics³³ and therefore retain an entirely closed amplicon consumable, including one that can be readily incorporated into a sample-in answer-out diagnostic system that is appropriate for point-of-care applications.

Disclosures

The authors declare no competing financial interests.

Acknowledgements

This work was supported by the National Institutes of Health (NIH) under grant RC3 AI089106.

MDR-TB nucleic acids were provided by the United Nations Children's Fund/United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases (TDR), Geneva, Switzerland.

We thank Dr. Tom Shinnick of the U.S. Centers for Disease Control and Prevention for guidance on the specific genes and mutations to include in the prototype assay.

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