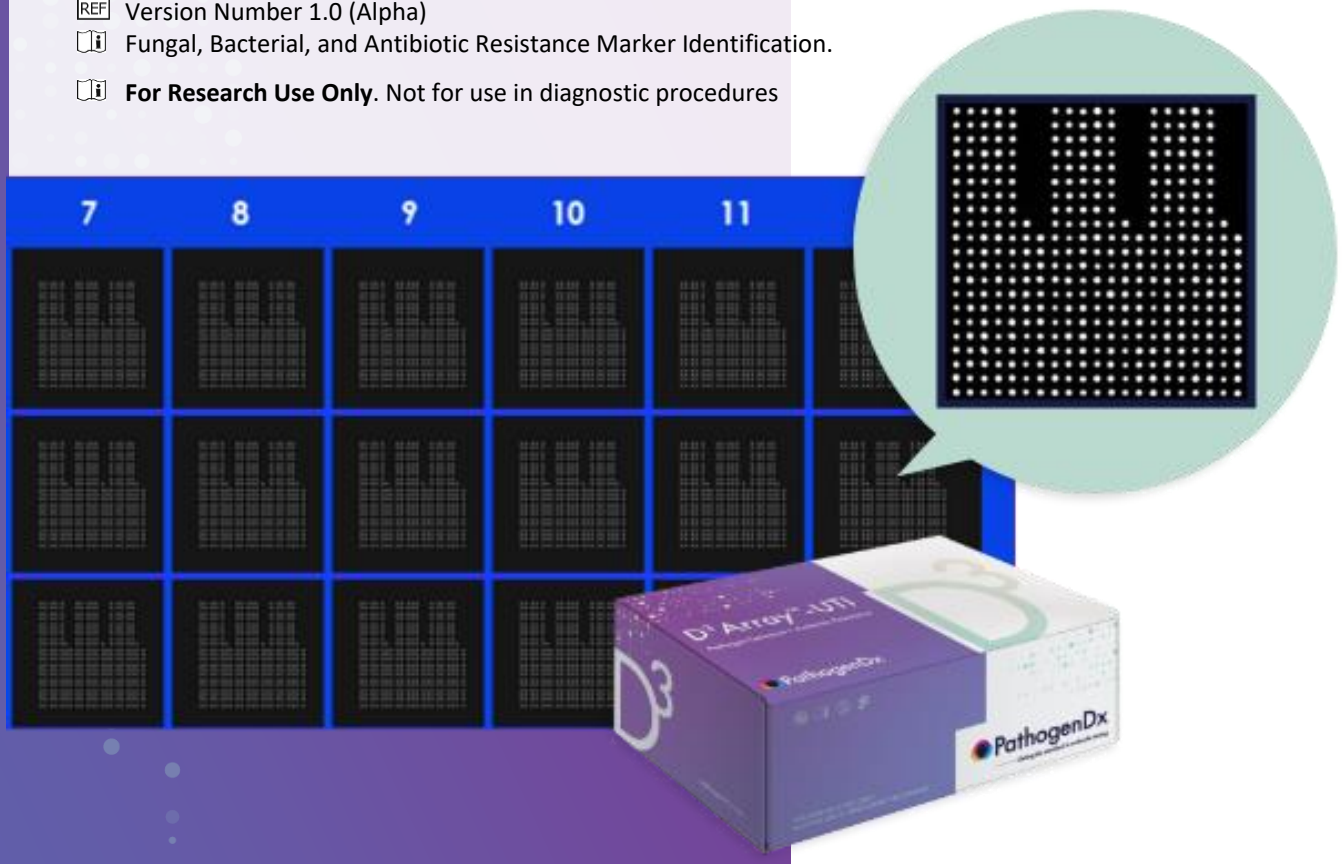
PathogenDx

PathogenDx Product Guide
D^3 Array™-UTI

REF Catalog Numbers: D^3 Array™-UTI (50-0067)
REF Publication Number:
REF Version Number 1.0 (Alpha)
Fungal, Bacterial, and Antibiotic Resistance Marker Identification.

For Research Use Only. Not for use in diagnostic procedures



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GENERAL INFORMATION

Intended Use

The D^3 Array™-UTI is a Research Use Only Assay intended for the semi-quantitative detection and identification of nucleic acids from bacteria and fungi, and detection of the antibiotic resistance gene markers associated with urinary tract infections (UTI). Positive results from the D^3 Array™-UTI are indicative of the presence of DNA derived from these pathogens. Positive results do not rule out an accompanying co-contamination with additional unregulated pathogens (microorganisms that are not typically targeted or regulated in the assay). Negative results do not rule out the presence of additional pathogenic organisms.

The D^3 Array™-UTI assay is intended for use by qualified and trained laboratory personnel specifically instructed and trained in the techniques of nucleic acid extraction, polymerase chain reaction (PCR), and microarray hybridization.

Summary and Explanation of the Test

UTI, or Urinary Tract Infection, is a common type of infection that can affect any part of the urinary tract system and drives antibiotic usage. UTI treatment has become complex because of increased antibiotic resistance. More broad-spectrum antibiotics are being used to treat UTIs, contributing to further antibiotic resistance. Inaccurate diagnosis and unnecessary treatment have also contributed to this situation. The detection and identification of urinary tract pathogens by molecular (PCR-Based) technologies has been shown to be more accurate than identification with culture-based approaches, and provide rapid, targeted, accurate identification of urinary tract pathogens as well as antibiotic resistance markers.

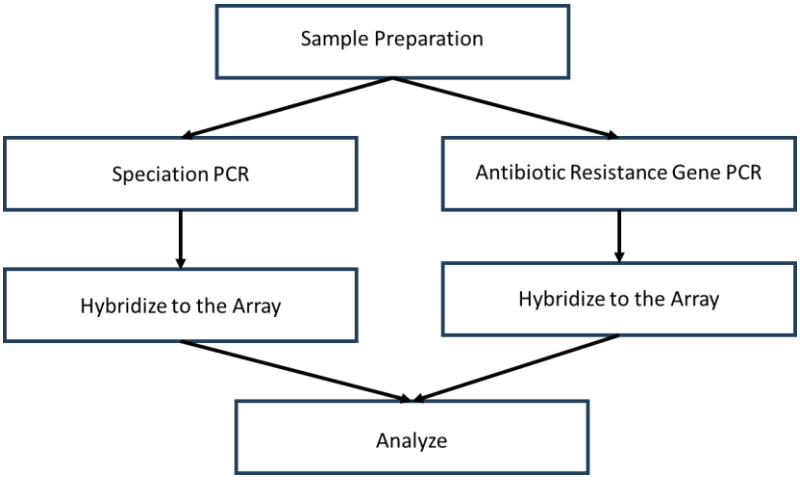
The D^3 Array™-UTI is a semi-quantitative test to detect the presence of bacterial organisms, fungal organisms, and antibiotic resistance gene markers in urine specimens. The test involves extraction of nucleic acids from urine samples followed by two PCR reactions, one for bacteria and fungi and one for antibiotic resistance gene markers. The PCR amplification product for each reaction is then hybridized to the array as two separate tests. Detection is determined by fluorescently labeled amplicon binding to specific spots in the microarray (regions of the array with complementary DNA), and then visualized using an automated fluorescence imager.

Principles of the Procedure

Overview

Nucleic acids are extracted from urine samples, from which a portion of the extracted material is removed and used to set up an asymmetric amplification that labels the targets and control target amplicons with a Cyanine 3 (Cy3) end-labeled primer. The fluorescently labeled PCR amplification products are directly hybridized to a DNA microarray printed on the bottom of each well in a 96-well plate. A plate reader will record the fluorescent intensity of the PCR product hybridized to specific spots (locations) on the microarray, and the resulting data from the plate image reader will then be uploaded to Augury™, PathogenDx's proprietary software for data analysis. Detection and identification of the presence of bacterial pathogens, fungal pathogens, antibiotic resistance markers, and positive control is automatically determined and reported using the Augury™ analysis software.

WORKFLOW



The **D³ Array™-UTI** process begins with a single sample preparation for each sample, and then PCR is performed separately for the detection and identification of urinary tract pathogens (speciation), and the antibiotic resistance markers. The PCR product derived from each of the two PCR reactions is then hybridized separately to the D³ Array™-UTI microarray plate.

Sample Preparation

The specimen required is purified DNA from urine samples. The PathogenDx D³ Array™-UTI is intended to be used only with purified DNA samples. Laboratories must validate their own DNA purification methodology. PathogenDx does NOT provide DNA purification kits or associated reagents.

The MAGMAX™ DNA Multi-Sample Ultra Kit catalog number A25597 (Thermo Fisher) has been validated for use on the D³-Array™-UTI and is recommended.

PCR Reaction Step

To perform the PCR step, approximately one-tenth **(5µL)** of the extracted nucleic acid sample is added to **45µL** of the PCR master mix associated with the detection and identification of urinary tract pathogens (Speciation) or Antibiotic Resistance Marker detection PCR reaction. Each reaction is then placed in a thermal cycler. The thermal cycling regime for the two reactions are identical. Consequently, both the Pathogen identification (Speciation) PCR and the Antibiotic Resistance Gene (ARG) marker PCR reactions may be performed at the same time in the same thermal cycler. The PCR functions to asymmetrically (preferentially) amplify the microbial targets and ARG. In the PCR reaction step, the target amplicons become labeled with the Cy3 fluorophore that is attached to the PCR amplification primers.

Hybridization Step - Hybridize amplified product to the microarray

The entire amplified PCR reaction is now ready for room-temperature hybridization to the DNA microarray without purification or heat denaturation steps. The **D³ Array™-UTI** DNA microarray is printed at the bottom of each well in a 96-well plate configuration. Each microarray contains all the necessary content to perform hybridization reactions to enable bacterial and fungal detection and species discrimination among all species of the D³ Array™-UTI pathogen target set. Each array also contains all probe content required to detect and resolve all Antibiotic Resistance Gene Markers of the D³ Array™-UTI assay. Thus, one plate can test and provide results for up to 46 samples plus one positive and one negative control sample. The 96-well plate microarray allows for the use of commonly used multi-channel, pipette-based, microplate processing for hybridization and all washing steps. This Product Insert includes protocols for both manual multichannel processing and automated processing using an Opentron OT-2 ™ liquid handling system. The automated protocol is configured to process the full 96-well microarray plate. The manual

method allows the user to utilize fewer columns based on the number of samples being run. The 96 well microarray plates are sealed and may be opened to accommodate only the wells to be used for analysis. Such sealing allows the use of only the wells necessary to manually process the required number of samples in any given run, saving the remaining wells (microarrays) for use in testing samples in subsequent runs or shifts.

Wash Step - Wash microarray and insert into plate image reader
Following a brief hybridization, the wells of the microarray plate are washed in a series of short rinses with PathogenDx wash buffer to remove unbound and unused Cy3 labeled primers or amplification products. After the final wash, the user ensures that the hybridized microarray plate wells have no residual wash buffer, and the dried plate is inserted into the Sensovation for imaging.

Plate Imaging Step - Plate reader performs analysis and interpretation of results
Each well in the 96-welll microarray plate contains a 21 x 21 matrix of DNA oligonucleotide probes printed in an ordered array on the bottom of the well. A portion of the oligonucleotides on each array are designed to identify specific regions of the bacterial and fungal pathogens included in the D^3 Array™-UTI assay to enable their identification (speciation). The second portion of the probes on the array are designed to identify markers within the Antibiotic Resistance Genes of the D^3 Array™-UTI assay to enable their individual detection. Also included in each array are probes used as external amplification controls, internal extraction controls, amplification controls and to provide a negative hybridization (background) signal.

The PCR products bound to specific target probes in the microarray will generate fluorescent signals, which are used to determine if target DNA is present in the original sample. The control data, obtained from the same microarray, are used to evaluate the specificity and validity of the hybridization data.

The fluorescent labeled hybridization signals from each microarray are scanned and analyzed using the Sensospot™ (Sensovation Inc) 96 well plate imager (plate image reader). The data are uploaded to a secured cloud-based server and automatically analyzed and interpreted using Augury™ software (PathogenDx Inc.). The results are reported to the user for assignment to sample records.

Data Analysis Step: Detection and Identification of Microbial Species and Antibiotic Resistance Genes
The D^3 Array™-UTI test performs two classes of nucleic acid analysis on a single 21x21 microarray

I. Microbial Species Detection and Resolution. The first function of the present D^3 Array™-UTI test is to detect and resolve a set of (24) bacterial species and (2) fungal "species of interest" using purified DNA extracted from each urine sample. See Figure 1 (Left Column) for the list of species. This is done by analysis of characteristic sequence changes that appear among a set of diverse, "Hypervariable" (HV) regions that occur within all bacterial and fungal ribosomal genes. In the present UTI assay, two bacterial (16S) HV regions (HV3, HV6) and one fungal region (28S) are interrogated in parallel (Figure 1, Top Row). Those HV regions are interrogated by first amplifying them all as a single multiplex, asymmetric PCR reaction along with a human positive control (RNase P) thus comprising a N=4 multiplex. The set of PCR primers used for such multiplex amplification are targeted to sequences which flank each HV region, and which are known to be common to all species of interest in the UTI assay. Thus, a single multiplex PCR reaction that is targeted to (3) discrete HV sites can amplify the entire set of (26) microbial "species of interest" in the present UTI assay.

Multiple species-specific microarray probes (at 4 per species) are designed to bind specifically within these HV regions. The numbered elements in Figure 1 describe each such species-specific probe. Each probe is printed in triplicate in the D^3 Array™-UTI. In that way, each bacterial or fungal species of interest is identified by (concurrent) sequence specific binding to 4 microarray probes, in triplicate. The resulting pattern of microarray probe binding is measured by Augury and is used to detect and resolve each species of interest. Because Augury analysis in the D^3 Array™-UTI test is based on 4 independent, sequence specific binding events, the resulting data becomes less

sensitive to experimental artifact and minimizes the risk of false positive misidentification of commensal microbes in the surrounding urinary microbiome.

II. Antibiotic Resistance Gene Detection and Resolution. The second function of the D^3 Array™-UTI test is to detect and resolve a set of (12) antibiotic resistance gene (ARG) "targets of interest" resident in the bacteria in a urine sample, along with a human positive PCR amplification control (RNase P). See Right Column of Figure 1 for the ARG's of interest. This is done in each sample by first amplifying them all as a single N=13 multiplex, asymmetric PCR reaction. The PCR primers used for such amplification are targeted to sequences which are unique to each ARG of interest in the UTI assay. Thus, a single multiplex PCR reaction can amplify the entire set of ARG targets in the present D^3 Array™-UTI test.

A single ARG-specific microarray probe is designed to bind specifically to each ARG that was amplified in the multiplex reaction. Subsequently, each ARG is identified by sequence specific binding to a unique cognate microarray probe, printed in triplicate. The resulting pattern of microarray probe binding is measured by Augury and used to detect and to identify which of the ARG targets of interest are presented in a urine sample.

Figure 1. The Species of Interest and Antibiotic Resistance Genes of Interest in the D^3 Array™-UTI test

	Bacteria								Funqi		ARGs
	16S rDNA HV3				16S HV6				28S rDNA		
	3A	3C	3E	PAN	16s.6a	16s.6c	16s.6e	16s.6g	1A	1B	
Aerococcus urinae	001	001	001	001	-	-	001	-	n/a	n/a	tetM
Acinetobacter baumannii	002	002	002	001	-	002	-	-	n/a	n/a	blaTEM
Citrobacter freundii	003	003	002	001	009	008	-	008	n/a	n/a	DFRA
Citrobacter koseri	022	022/023	004	001	004	004	-	-	n/a	n/a	mecA
Enterobacter (Klebsiella) aerogenes	005	005	002	001	009	008	-	008	n/a	n/a	Gyrase A
Enterobacter cloacae	022	023	004	001	004	005	-	021	n/a	n/a	blaSHV
Enterococcus faecalis	006	006	003	001	-	-	001	-	n/a	n/a	aac6-1b
Enterococcus faecium	007	007	003	001	-	-	007	-	n/a	n/a	blaACT
Escherichia coli	004	004	004	001	004	004	-	-	n/a	n/a	blaCTX
Klebsiella oxytoca	024	024	004	001	009	008	-	008	n/a	n/a	VanA
Klebsiella pneumoniae ATCC13883	025	025	004	001	004	005	-	021	n/a	n/a	mcr-1
Klebsiella pneumoniae ATCC13884	026	026	004	001	004/009	005/008	-	021	n/a	n/a	blaOXA-48
Klebsiella pneumoniae ATCC11296	005	005	004	001	009	008	-	021	n/a	n/a	
Morganella morganii	008	008	005	001	009	008	-	008	n/a	n/a	
Proteus mirabalis	009	009	004	001	009	009	-	-	n/a	n/a	
Proteus vulgaris	010	010	004	001	009	009	-	-	n/a	n/a	
Providencia stuartii	011	011	005	001	009	011	-	008	n/a	n/a	
Pseudomonas aeruginosa	012	012	006	001	-	012	-	-	n/a	n/a	
Staphylococcus aureus	014	014	007	001	-	014	-	-	n/a	n/a	
Staphylococcus saprophyticus	015	015	007	001	-	015	-	-	n/a	n/a	
Streptococcus agalactiae	016	016	008	001	-	016	-	-	n/a	n/a	
Serratia marcescens	017/27	017	002	001	009	017	-	008	n/a	n/a	
Mycoplasmoides genitalium	018	018	009	001	-	018	-	-	n/a	n/a	
Metamycoplasma hominis	019	019	010	001	-	019	-	-	n/a	n/a	
Ureaplasma parvum	020	020	011	001	-	021	-	-	n/a	n/a	
Ureaplasma urealyticum	020	020	011	001	-	020	-	-	n/a	n/a	
Candida albicans	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	001	001	
Candida glabrata	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	001	002	

REAGENTS AND EQUIPMENT

PathogenDx Kit Components

- **PCR Master Mix:**
  - **PCR Master Mix (**30-0876) containing Molecular Biology Grade Water, PCR Buffer, $MgCl_2$, BSA and dNTPs including dUTP for PCR amplification
  - **Pathogen Detection and Identification (Speciation) Primer Set (**30-0869) for amplification and fluorescent labeling of the speciation PCR product
  - **ARG Primer Set (**30-0868) for amplification and fluorescent labeling of the set of antibiotic resistance gene PCR products
  - **Taq Polymerase with Cod UNG (**30-0877) catalyzes the DNA template dependent DNA synthesis and includes Cod UNG for eliminating possible carry-over amplicon contamination
  - **Universal Array Control (not included)** will be added to the next version not in the present version of the array

- **Hybridization and Analysis**
  - **PathogenDx microarrays** (30-0866) with sequence-specific oligonucleotide probes attached to the surface and a Cy5 dye co-print for accurate grid alignment of the spots for subsequent imaging. The 96 well plate is provided with a barcode number of which the first 4 digits correspond to the probe map for the specific content of the **D³ Array™-UTI** and the last 6 digits are the unique lot and print identifier for the 96 well plate being used.
  - **Buffer 1 (**30-0087) for binding of target DNA to sequence-specific oligonucleotide probes
  - **Buffer 2** (30-0088) for equilibration of arrays and optimal binding of target DNA to sequence specific oligonucleotide probes
  - **Augury©** software for automated microarray analysis and fungal and bacterial profile identification

Equipment Required (but not provided)

- The following equipment and software are required to run the test and analyze results.
- Substitutions must be validated by the user.
- All equipment must be calibrated according to the manufacturer's specifications. It is the responsibility of the laboratory to maintain calibrated equipment.
- Additionally, the equipment required for sample purification is the responsibility of the laboratory and not listed in this document as it will be dependent on the sample purification methodology chosen by each lab.

Equipment	Number Required	Vendor	Catalog #
Sensovation Sensospot	1	Sensovation	Sensospot
Computer Keyboard and Mouse	1	Amazon	B017M4J1BU
Barcode Scanner	1	Amazon	B00LE5VV1C
MiniAmp Thermal Cycler	1	ThermoFisher Scientific	A37834
Fisherbrand Microplate Centrifuge	1	Fisher Scientific	14-955-300
Mini Centrifuge w/ 8 Place Tube Rotor	1	Fisher Scientific	NC0990506
Fisherbrand Analog Vortex Mixer	2	Fisher Scientific	02-215-414
MicroAmp Optical Film Compression Pad	1	Applied Biosystems	4312639
Plate-Sealing Paddle	1	Fisher Scientific	NC1613699
Augury™ Software (Version 4.8.10)	N/A	PathogenDx	N/A
Opentrons OT-2	1	Opentrons	OT-2 Robot
Dedicated laptop for Opentrons OT-2 Robot	1	N/A	N/A
Pipettors		**Vendor**	**Catalog #**
Fisherbrand Elite Pipettors 1 to 10µL	1	Fisher Scientific	FBE00010
Fisherbrand Elite Pipettors 2 to 20µL	1	Fisher Scientific	FBE00020
Fisherbrand Elite Pipettors 20 to 200µL	1	Fisher Scientific	FBE00200
Fisherbrand Elite Pipettors 100 to 1000	1	Fisher Scientific	FBE01000
Fisherbrand Elite Multichannel Pipette 1 to 10µL	1	Fisher Scientific	FBE800010
Fisherbrand Elite Multichannel Pipette 10 to 100µL	1	Fisher Scientific	FBE800100

Consumables Required (but not provided)

- The consumables listed below are for the PathogenDx assay and do not include the consumables required for sample purification.

Part Description	Vendor	Catalog #
10µL barrier tips	USA Scientific	1121-3810
20µL barrier tips	USA Scientific	1120-1810
200µL barrier tips	USA Scientific	1120-8810
1000µL barrier tips	USA Scientific	1126-7810
5mL sterile tubes	USA Scientific	4011-9487
1.5mL tubes	Costar	3207
Disposable pipette basins 10mL	Fisher Scientific	50-187-1120
Disposable pipette basins 50mL	Fisher Scientific	50-112-7246
Kimwipes Delicate Task Wipers	Fisher Scientific	06-666A
AlumaSeal II	Genesee Scientific	12-169
0.2 ml 96-well PCR Plate	Genesee Scientific	27-108
Molecular Biology Grade Water– DNase/RNase Free	Fisher Scientific	BP24701
Nunc Sealing Tape	Fisher Scientific	276014
15mL polypropylene sterile tubes	Fisher Scientific	12-565-268
50mL conical polypropylene sterile tubes	Fisher Scientific	12-565-270
Opentrons OT-2 Filter Tips, 200µL	Opentrons	999-00081
USA Scientific Inc 12-channel reservoir for automation	Fisher	NC9056368
300 mL disposable trough	Fisher	30077313

The materials listed above have been fully tested and are recommended for this platform. The use of alternative materials must be validated by the customer.

Warnings and Precautions ⚠

- **Warning:** This product is for Research Use Only. Not for use in Diagnostic Procedures.
- **Caution:** Primer Sets are light sensitive and must be stored away from light.
- **Caution:** PathogenDx microarrays are light and moisture sensitive and should be stored in the moisture barrier bag with desiccant packet provided with the kit.
- **Caution:** In the post-hybridization protocol, centrifuge speed should not exceed 70xg, or the plates may break.
- **Caution:** Refer to the Safety Data Sheets
- **Caution:** Kit components from different kits must NEVER be mixed. Once all the wells on the 96 well plate have been used, discard all reagents associated with this plate. **PathogenDx does not support or sell partial kits.**

Storage Instructions

- **PCR Master Mix** should be stored at -20°C± 5˚C and may be freeze-thawed up to 6 times.
- **Speciation Primer Set** should be stored at -20°C± 5˚C and may be freeze-thawed up to 6 times and must be protected from light.
- **ARG Primer Set** should be stored at -20°C± 5˚C and may be freeze-thawed up to 6 times and must be protected from light.
- **Taq Polymerase with Cod UNG** should be stored at -20°C± 5˚C. Taq Polymerase is thermostable and does not need to thaw, only remove from freezer when starting PCR process.
- **Universal Array Control (not available)**, it will be added to a future version of the assay.
- **Buffer 1** should be stored at room temperature.

- **Buffer 2** should be stored at -20°C± 5˚C and may be freeze-thawed up to 6 times.
- **PathogenDx Microarrays** are provided in a moisture barrier bag with desiccant. The plates should be stored in this manner to protect them from light and moisture. Unused wells **MUST** remain covered until ready for use. Store at room temperature in the moisture barrier bag provided to protect from light.

Instability

- If salt precipitation has occurred in Buffer 1 after shipping or prolonged storage, re-dissolve by vortex mixing at room temperature (20-25°C).
- If there is no detectable Cy5 signal on the PathogenDx microarrays pre or post-hybridization there may have been bleaching during storage or imager settings are not correct. If needed, please refer to the Troubleshooting Guide in this product insert.
- If PCR did not take place, the Taq Polymerase may have been activated by excess heat during PCR setup. The enzyme is inactive at +15 to +25°C during PCR setup, and then activated at +95°C during initial denaturation.

Work Areas and Best Practices

(See Appendix A for Details) Assay Procedure:

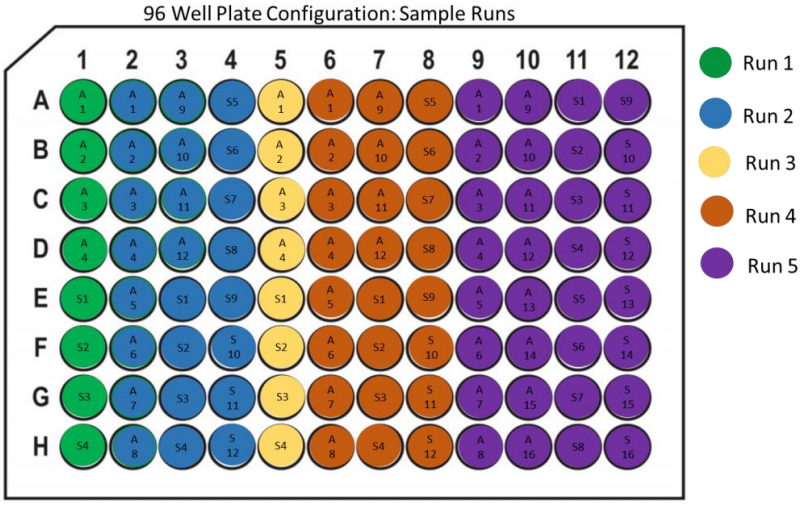
Process Overview:

The image below shows the configuration of a 96 well plate where position A1 refers to Well 1 and position H12 refers to well 96.

96 Well Plate Configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

- A 96 well plate can run 48 samples in a single run (48 samples for ARG and 48 samples for pathogen detection and identification (Speciation)). We recommend using at least one well for an NTC (No Template Control) and one well for a positive control, however this is performed at the laboratory's discretion.
- If an entire plate is not used for a single run, the unused wells MUST be kept covered until ready for use to avoid any contamination or wetting. If wetting occurs in unused wells, they may not be used in future experiments!
- The covered wells will not affect the user's ability to analyze uncovered wells.
- The configuration of the samples must be followed for the software to generate accurate reports. For the current automated hybridization protocol on the Opentrons OT-2, a full plate must be used.
- For manual hybridization, an example of a plate with multiple runs is shown below:



- Unused wells must remain covered until ready for use.
- In the image, 4 runs have been performed over 4 different days where varying numbers of samples are used (ABR designated by A and Speciation designated by S).
- Note each run is set up for one or more columns to be compatible with Opentrons software.

Sample Preparation:

1. Laboratories are responsible for their own sample extraction procedures. PathogenDx does NOT provide sample extraction kits or reagents.
    a. The MAGMAX™ DNA Multi-Sample Ultra Kit catalog number A25597 kit has been validated on the D³ Array™-UTI and is recommended. Extracted samples should be stored according to the kit manufacturer's instructions.

PCR amplification (set up in pre-amplification area)

1. Thaw PCR Master Mix and both Primer Sets. Two master mix tubes will be made: one for ARG and one for pathogen detection and identification (Speciation). **The Taq Polymerase is thermostable and does not need to thaw, only remove from freezer when starting the PCR process**).
2. Use Table 1 to calculate the appropriate volumes needed for the reactions.
    a. Please note the following table includes volumes for multiples of 8 which corresponds to the number of wells in a 96 well plate column. Additional volume has been included to account for pipetting loss.
    b. For a full 96 well plate, 2 tubes of master mix will be made: one for 48 reactions for ARG and a second master mix for 48 reactions for pathogen detection and identification (speciation).
3. Vortex all reagents except the Taq polymerase for 15 seconds; centrifuge at 1000xg for 3-5 seconds.
4. Mix the indicated reagent volumes (calculated from Table 1) in a sterile tube to prepare the PCR Master Mix (MUST be made fresh each run.)

Table 1: PCR Reaction Volumes

Number of Reactions	PCR MasterMix (µL)	Primer Set (µL) Speciation or ARG	Taq Polymerase with cod UNG (µL)	Total Volume (µL)
1	41.6	2	1.4	45
8	416	20	14	450
16	748.8	36	25.2	810
24	1081.6	52	36.4	1170
32	1497.6	72	50.4	1620
40	1830.4	88	61.6	1980
48	2163.2	104	72.8	2340

5. Briefly vortex the PCR master mix for 5 seconds and then centrifuge at 1000xg for 3-5 seconds.
6. Store all reagents at -20°C after use.
7. Pipette 45µL of the PCR Master Mix into the designated PCR strip tubes or wells of a PCR plate.
8. In the Sample Prep Area, pipette 5µL of the purified sample prep into the corresponding tube or well for a final volume of 50µL per PCR reaction. Pipet up and down to mix.
9. **Always check pipette tip volumes before and after to ensure accuracy and release.**
10. Cap tubes, or seal plates with PCR film ensuring every well is completely sealed.
11. Centrifuge at 1000xg for 3-5 seconds.
12. Place tubes or plate into the thermal cycler with a pressure pad if necessary, before closing the thermal cycler lid. It is crucial to ensure that all PCR tubes or plates are firmly mounted in the thermal cycler's 96 well block.
13. Refer to Table 2 to run the PCR Program.
14. PCR product may be stored for up to 1 day at room temperature protected from light.

Table 2: PCR program

Steps	Temp.	Time	Cycles
1	25°C	5 minutes	1
2	95°C	4 minutes	1
3	95°C	30 seconds	45
4	55°C	30 seconds	
4	72°C	30 seconds	
6	72°C	5 minutes	1
7	25°C	∞	1

Hybridize PCR Amplified Product to Microarray-Opentrons Full Plate Method

CAUTION: Avoid contact with the entire bottom surface (outside glass plate) of the microarray 96-well plate during processing as this may adversely affect the clarity of the image acquisition. Use only the 96-well plate edges or barcoded area for handling.

CAUTION: Do not allow the microarray wells to air dry. As soon as the Opentrons protocol is complete, immediately proceed to plate centrifugation.

CAUTION: Avoid contacting the microarray surface with the pipette tip(s).

Prepare the 96-well microarray plate for hybridization

1. Perform all steps in the Hybridization/Post PCR Area, following standard unidirectional flow for PCR work.
2. Before starting, thaw Buffer 2 at room temperature and mix by vortexing prior to use.
3. Prepare the Pre-hybridization Buffer, Hybridization Buffer, and Wash Buffer in sterile appropriately sized tubes or bottles certified DNase-free using Tables 3, 4, and 5.
4. Vortex Pre-hybridization Buffer, Hybridization Buffer, and Wash Buffer before use.

Table 3: Pre-Hybridization Buffer

Pre-hybridization Reagents	Volumes for 96-well Run on Opentrons
Molecular Biology Grade Water (mL)	13.67
Buffer 1 (mL)	4.089
Buffer 2 (mL)	2.15

Table 4: Hybridization Buffer

Hybridization Reagents	Volumes for 96 Wells
Buffer 1 (mL)	1.227
Buffer 2 (mL)	0.645

Table 5: Wash Buffer

Wash Buffer Reagents	Volumes for 96-well Run on Opentrons
Buffer 1 (mL)	0.6
Molecular Biology Grade Water (mL)	79.40

5. Remove the PCR plate from the thermocycler and briefly centrifuge.
   CAUTION: **care should be taken during these steps to avoid well to well cross-contamination**
6. Carefully remove the adhesive cover from the plate.
7. Add **18µL** of Hybridization Buffer to each well of the PCR plate, gently pipette up and down to mix 7-10 times. The PCR product and the Hybridization Buffer mix constitute the Hybridization Cocktail.
   a. Always dispense and evacuate in the well to avoid aerosolizing the PCR product or contaminating neighboring wells.
   b. Ensure no bubbles form near the bottom of the wells during mixing.

Setup the Opentrons OT-2 Deck to Perform the Hybridization and Washes

1. For the full plate set up, place the following labware on the deck of the OT-2 (Figure 1) according to the labware layout (Figure 2).

Figure 1. Opentrons Deck

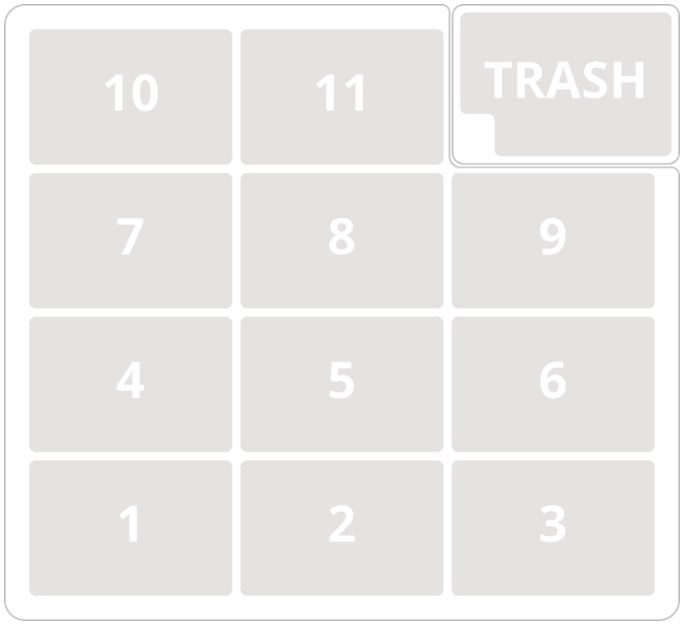
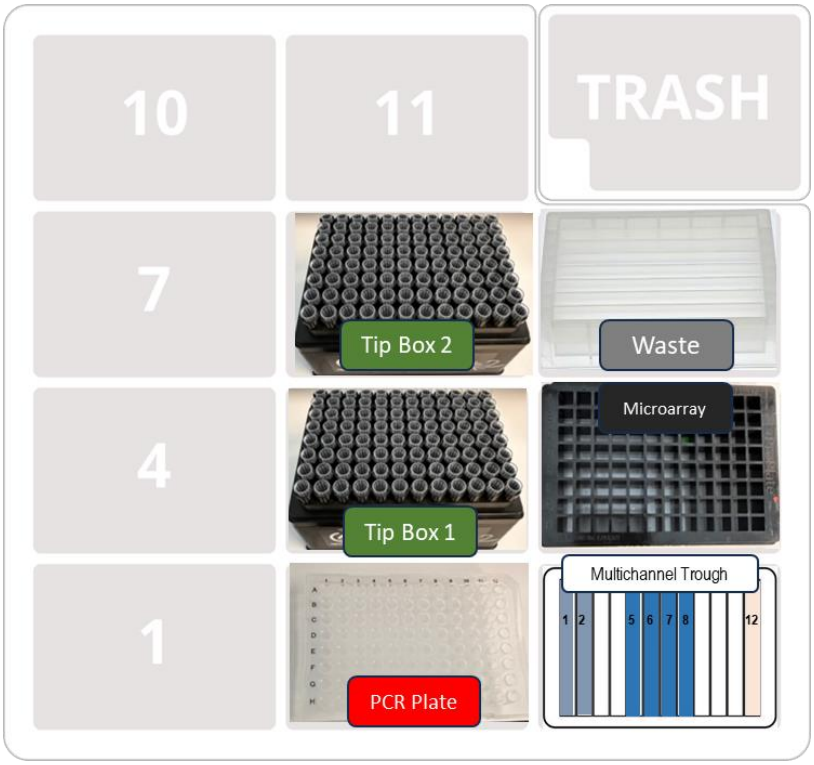


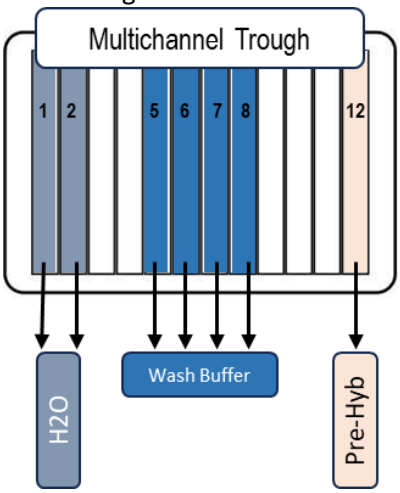
Figure 2. Labware Arrangement on the Opentrons OT-2 Deck



a. One- 300 mL Disposable Trough (Waste) (position 9)
b. UTI Microarray Plate (position 6)
c. One- 12-Channel Reservoir (position 3)
d. Two- Opentrons OT-2 Filter Tips, 200 µL (positions 5 and 8)
e. The PCR plate containing the Hybridization Cocktail (position 2)
   Note: It is highly recommended that the deck assembly is performed from back to front.

2. Ensure all components are properly placed on the deck, firmly secured within their slots.
3. Add 18 mL of H_2O (Molecular Biology Grade), Wash Buffer and Pre-Hyb Buffer to their designated channels in the 12 Channel Reservoir (Figure 3).
   a. Note: the buffers should be added to their respective channels prior to placing the 12-Channel Reservoir on the deck.

Figure 3. Buffer Arrangements in Multichannel Reservoir



4. Close the Opentrons door.
5. Launch the Opentrons software, select the OT-2 protocol "12 Column Hyb UTI v4.0.2" and start the run. Note: Ensure that the instrument's internal lighting remains OFF during the run.
6. Set up a timer for 2 hours and 47 minutes.
7. Immediately after the run is complete, remove the Microarray plate and spin dry using the plate centrifuge for 2 minutes.
   a. After centrifugation, remove the plate and inspect for any remaining moisture. If moisture is present, repeat the centrifugation step until completely dry.
8. Prior to scanning, clean the back of the glass microarray with lens paper or Kim wipe (never use paper towels which leave an excess of fibers and interferes with scanning).
   a. If the back of the slide still shows dust and/or streaks, lightly spray a Kim wipe with 70% ethanol and wipe the back of the plate dry.
9. PathogenDx plates should be placed back into the moisture barrier bag with desiccant until scanning may be performed to protect the arrays from light. Once placed with desiccant in the dark, plates should be scanned within two weeks of hybridization.

Opentrons Post-run Cleanup

1. Remove the tip waste container and dispose of the tips appropriately.
2. Wipe down the interior of the tip waste container with 10% bleach, followed by 70% EtOH before placing it back on the Opentrons deck.
3. Remove the liquid waste trough and treat with 10 mL of beach for 10 minutes prior to disposing.
4. Remove and discard remaining used labware.
   a. Note: pipette box 2 which is partially used can be saved and used for a second run, rotating it prior to placing on the Opentrons.
5. Wipe down the surface of the OT-2 deck with 70% EtOH.
6. Exit the Opentrons software.

Scanning the Microarray and Data Acquisition

1. Access the Sensovation scanner desktop, select the application "Array Reader".
2. Open the tray, select "Open Tray".
3. Place the microarray into the tray oriented with the plate face up and aligned with A1 in the top left corner.
4. Close the tray, select "Close Tray".
   a. NOTE: ***NEVER force the plate holder shut by hand.***
5. Select "Scan".
6. From the dropdown menu for "Rack Layout" select the Full Slide (96 wells) PDx.
7. From the dropdown menu for assay layout, select "3FlashGreen".
8. Click on the three dots icon to the right of "Scan Position".
9. To scan a full plate, double click the asterisk at the top left of the plate image.
10. To scan a partial plate, click the desired wells or click on the column number.
    a. **Note:** ***All other information on this screen is preprogrammed – do not alter.***
11. Select the Blue Arrow to begin the scanning process.
12. While the plate is being scanned, select "Result Overview" to review the images of the wells.
13. When the plate is finished scanning and the screen displays the digital image of a plate with all green wells, select the Red X to exit the scanning process.
14. Open the tray, select "Open Tray".
15. Remove the microarray and store inside the moisture barrier bag with the desiccant packets.

16. Close the tray, select “Close Tray”.
17. Exit the Array Reader application, select “Exit”.
18. On the Sensovation Scanner desktop, select the folder “Scan Results”.
19. Locate the folder associated with your plate and rename the folder with the plate barcode number by scanning the barcode located either on the outside of the barrier bag or on the plate itself.
    a. Rename the scan file to reflect the plate barcode. For example, rename “ScanJob-191108130334_1” to “1317001001” and add the wells scanned to the end of the barcode.
    b. For example, if the first two columns were scanned rename “ScanJob-191108130334_1” to “1309001001.well001-well016”.
    c. When identifying which wells to analyze, always use the format:
        1. <barcode>.well<aaa>-well<bbb> *no spaces or other characters present*
        ii. where aaa and bbb are always written as 3-digit numbers.
20. Submit the whole barcode labeled folder to Portal.

QUALITY CONTROL AND VALIDITY OF RESULTS

1. One Negative Control (No Template Control) and one External Positive Control should be processed with each run.
2. The positive process control ensures that the isolation and amplification of the nucleic acids was successful. The No Template Control ensures that there were no systematic errors introduced during the isolation and amplification of the nucleic acids.
3. Validation of results is performed automatically by the Augury™ Software based on the performance of internal positive and negative controls in each well.

INTERPRETATION OF RESULTS (VERSION 1.0)

Table 6: Interpretation of Results (Pathogen identification/Speciation)

Reporting Probe	Sample Report: Negative Sample	Sample Report: Positive Sample	Sample Report: Invalid Result	Negative Control: Valid Result	Negative Control: Invalid Result
Negative Control	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
RNase P	**Detected**	**Detected**	**Detected**	Not Detected	**Detected**
Candida albicans	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Candida glabrata	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Acinetobacter baumannii	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Aerococcus urinae	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Citrobacter freundii	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Citrobacter koseri	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Enterobacter aerogenes	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Enterobacter cloacae	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Enterococcus faecalis	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Enterococcus faecium	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Escherichia coli	Not Detected	**Detected**	**Detected**	Not Detected	**Detected**
Klebsiella oxytoca	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Klebsiella pneumoniae	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Morganella morganii	Not Detected	**Detected**	**Detected**	Not Detected	**Detected**
Mycoplasma hominis	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Mycoplasma genitalium	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Proteus mirabilis	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Proteus vulgaris	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Providencia stuartii	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Pseudomonas aeruginosa	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Serratia marcescens	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Staphylococcus aureus	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Staphylococcus saprophyticus	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Streptococcus agalactiae	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Ureaplasma parvum	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Ureaplasma urealyticum	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Status	**Valid**	**Valid**	**Invalid**	**Valid**	**Invalid**
Results	Sample is Negative for the listed organisms	Sample is POSITIVE for the listed organisms	Results are invalid	Negative Control Run Successfully	Results are invalid
Action	Review regulations and report accordingly	Review regulations and report accordingly	Perform a retest	Negative Control should be recorded	Perform a retest

Table 7: Interpretation of Results (ARG)

Reporting Probe	Sample Report: Negative Sample	Sample Report: Positive Sample	Sample Report: Invalid Result	Negative Control: Valid Result	Negative Control: Invalid Result
Negative Control	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
RNase P	**Detected**	**Detected**	**Detected**	Not Detected	**Detected**
tetM	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
blaTEM	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
dfrA	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
mecA	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
GyrA	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
blaSHV	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
aac6-Ib	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
blaACT	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
CTX	Not Detected	**Detected**	**Detected**	Not Detected	**Detected**
VanA	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
mcr-1	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
blaOXA	Not Detected	Not Detected	**Detected**	Not Detected	**Detected**
Status	**Valid**	**Valid**	**Invalid**	**Valid**	**Invalid**
Results	Sample is Negative for the listed organisms	Sample is POSITIVE for the listed ARG	Results are invalid	Negative Control Run Successfully	Results are invalid
Action	Review regulations and report accordingly	Review regulations and report accordingly	Perform a retest	Negative Control should be recorded	Perform a retest

Figure 4: Sample Report for a single speciation well from the Type2.csv Report

Well Number:	83		
Sample Name:	Sample #83		
** Controls: **			
	Negative Control	Not Detected	
	RNaseP Control	Detected	
** Pathogens/Genes Detected: **			
		cells/ml	Bin
	Klebsiella pneumoniae	1.8M	1M - 10M
	Enterobacter/Klebsiella aerogenes	620k	100k - 1M

In this figure, the sample in well 83 was tested and found to have 2 organisms present. If an organism is found to be Present or Possibly Present it is listed in the Type2.csv report. The Bin lists the range of cells/mL to which the organism is found, and the cells/mL column shows an actual count. In this example, *Klebsiella pneumoniae* was determined to be present with a cell/mL value of 1.8 million. The sample was also found to have *Enterobacter/Klebsiella aerogenes* present at a cell/mL value of 620,000. The Negative Control was determined to be Not Detected, as expected. Likewise, the RNase P Control was detected, as expected.

Figure 4: Sample Report for a single ARG well from the Type2.csv Report

Well Number:	2		
Sample Name:	Sample #2		
** Controls: **			
	Negative Control	Not Detected	
	RNaseP Control	Detected	
** Pathogens/Genes Detected: **			
		cells/ml	Bin
	tetM	N/A	N/A
	blaTEM	N/A	N/A

In this figure, the sample in well 2 was tested and found to have 2 antibiotic resistance genes present. If an ARG is found to be Present or Possibly Present it is listed in the Type2.csv report. The Bin and the cells/mL column are listed as N/A since the quantifiable portion of the assay only applies to speciation. In this example, tetM and blaTEM were found to be present in the sample. The Negative Control was determined to be Not Detected, as expected. Likewise, the RNase P Control was detected, as expected.

LIMITATIONS OF THE PROCEDURE

- The assay is intended for use by qualified and trained laboratory personnel specifically instructed and trained in the techniques of nucleic acid extraction, PCR, and microarray hybridization.
- A thorough understanding of the instructions for use is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following these instructions for use.
- If state or local health authorities require, laboratories must report all positive results to the appropriate authorities. Typically, UTI results do not require reporting but check your local requirements for information.
- Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the target sequences.
- The MAGMAX™ DNA Multi-Sample Ultra Kit catalog number A25597 kit has been validated for use with the **D3-Array-UTI** and is recommended for laboratories to use. If other sample purification kits or systems are used, they must be validated by the user.
- False-negative results may arise from:
    - Improper sample collection
    - Specimen collection after nucleic acid can no longer be found in the specimen matrix
    - Using unauthorized extraction or assay reagents
    - The presence of PCR inhibitors
    - Failure to follow instructions for use
- False-positive results may arise from:
    - Cross-contamination during specimen handling or preparation
    - Cross-contamination between samples
    - Specimen mix-up
    - DNA contamination during product handling
- It is recommended to run a no template negative control (NTC) as well as a positive control to ensure no contamination has occurred. If hybridization signal is detected in the NTC, new reagents should be used.
    - The primers and the PathogenDx plates are light sensitive and should be protected to avoid photobleaching.

- All equipment should be properly calibrated according to the manufacturer's guidelines including yearly maintenance, if required.

PATHOGENDx TROUBLESHOOTING GUIDE

Issue observed	Possible cause	Resolution
GENERAL TROUBLESHOOTING		
REAGENTS		
Salt precipitation occurs after rehydration and prolonged storage of Buffer 1	Salt concentration in hybridization cocktail will be too low	Re-dissolve by vortex mixing at room temperature (20-25°C)
Photobleaching of Primer Set	Primer Set contains CY3 and can photo bleach with extended exposure to light	Keep Primer Sets in a tinted tube and do not allow prolonged light exposure
NO SIGNAL IS DETECTED		
	PCR Setup Error/Incorrect Primer Sets used	Repeat experiment and carefully check to ensure all reagents are added in the correct volumes. Carefully note which wells belong to which samples and primer sets.
	Taq polymerase may have been activated by excess heat during setup. The enzyme is inactive at 15-25 °C and active at 95°C.	Repeat experiment with new reagent.
	Insufficient Template: purified sample was not transferred to Master Mix plate	Rerun PCR, be sure to pipette up and down to mix and check tips for full evacuation.
	New plastics (i.e. PCR plates, seals) are inhibiting PCR or causing evaporation	Check catalog number of plates; run QA on any new materials being used.
	Sample evaporation	Check the wells in the plate after thermal cycling and centrifugation. Ensure high quality seals and plates are used. If a pressure pad is required for the thermal cycler, ensure it is placed correctly on the plate. Always seal the plate carefully with a seal applicator.
	Thermal Cycler conditions have been changed	Thermal Cycler conditions should match provided PCR cycling program. Conditions differ between PathogenDx kits.
	PCR plate not spun down	After application of seal, spin down PCR plates to ensure all DNA and reagents are contained at bottom of plate.
	PCR plates being left out before being set in Thermal Cycler	After application of seal, spin down and immediately place in Thermal Cycler to run PCR program.
	Photobleaching of Primer Set due to light exposure	Repeat PCR with a new Primer Set.
SIGNAL IS DETECTED IN NEGATIVE CONTROLS		
Background Signal is high	Post-hybridization wash was not stringent due to incorrect dilution of wash buffer	Prepare wash buffer again and repeat hybridization with new slides.
	Contamination	Decontaminate all areas with 10% bleach then 70% ethanol. Replace all consumable items. Sterilize pipettes. Always use filter/barrier tips. Sterilize equipment. Ensure materials, pipettes, and equipment are dedicated to each area and never transferred to other areas. Follow all protocol and notes listed within the Product Insert.

Issue observed	Possible cause	Resolution
	Contaminated template added to NTC	Run the NTC as Master Mix ONLY, do not add a template.
Non-Specific Amplification OR Positive Detection in Negative Controls	Contaminated Reagents	Use a fresh aliquot of reagents. Confirm the NTC is clean.
	Aerosol Contamination	Use filter/barrier tips for all pipetting. Ensure centrifugation steps are being performed prior to pipetting, refer to the Product Insert. Change gloves frequently and open and close tube lids carefully. Remove seals from PCR plates carefully.
	Pipet tips not changed between samples	Always change tips between samples. When in doubt if a tip has been contaminated, discard and use a new pipet tip.
	Aspiration Flask/Container is full and causing liquid to backflow into the pump.	Waste must be discarded before the liquid level reaches the hose attachment. Dispose of carefully in an area removed from PCR prep or sample prep. Always bleach the sink and any contact surfaces after disposal. The waste container may also have 10% bleach added to it to reduce risk of amplicon contamination during disposal.
PCR TROUBLESHOOTING		
Suspected PCR Failure	PCR reagents were not thawed completely	Allow to thaw completely and rerun samples.
	PCR Master Mix and reagents were not vortexed before use	Always vortex and briefly centrifuge reagents before use.
HYBRIDIZATION TROUBLESHOOTING		
Suspected Failure during Hybridization	Photobleaching of microarrays due to incorrect storage	Obtain a new PathogenDx slide that has been stored correctly.
	Bleach vapors in the Hybridization Chamber are causing slide bleaching	Hybridization Chambers may be cleaned with 10% bleach but then MUST be wiped down thoroughly and allowed to dry completely before use. The chamber must be left open to dry completely and allow vapors to dissipate.
	Hybridization wash concentration was altered	The concentration MUST not be changed. Increasing or decreasing Buffer 1 will cause increased background or no signal respectively.
	Pre-Hybridization and Hybridization buffers were premade and left out or frozen overnight	Buffers should only be made during Hybridization process, make fresh buffers, and rerun.
	Did not add hybridization buffer to DNA product	Remake PCR plate and repeat hybridization.
	Did not pipette up and down to mix hybridization buffer into PCR product	Remake PCR plate and repeat hybridization.
	Wash Buffer was made incorrectly	Follow table provided to make wash buffer in correct volumes.
	Aspirator manifold is being washed with bleach or ethanol and used without adequate rinsing	If using either to decontaminate the manifold, adequate rinsing of 100mL MB Grade Water is required to completely remove residue.
	Pipet basins are being washed with bleach and reused	Utilize sterile pipet basins for each hybridization.

Issue observed	Possible cause	Resolution
	Aspirations were inadequate during wash steps	Use the recommended aspiration set-up.
	Popping bubbles left in liquid on slides	Do Not pop any bubbles that may be on the liquid on the slides. This will cause cross contamination or removal of probes.
	Dust or fibers were present on slide	Use Kimwipe to clean the back of the slide.
	Compressed air was used to remove fibers from slides	Compressed air must not be used on slides at any time. Residue is often deposited that may affect the integrity of the probes
MICROBIOLOGY TO MOLECULAR BIOLOGY TROUBLESHOOTING		
Results are not as expected	Reported values do not match expected results	Companion plating should be performed to verify results. Not all organisms found in urine will grow on certain plate types. These organisms may be detected by the PathogenDx platform resulting in higher values.
	Fractional Recovery was observed	Fractional recovery at a low CFU level is expected.
	My spiked samples failed to be detected	Check the companion plates to ensure the dilution into the sample prep is detectable by the assay. If the spiking concentration is approaching the limit of detection, at least 10 replicates should be performed and fractional recovery should be observed.
SCANNING TROUBLESHOOTING		
Imager	Sensovation settings were changed	Refer to Sensovation user manual to reset settings and contact PathogenDx Technical Team for further instruction.
	Sensovation imager was dropped, jostled, misaligned etc.	Refer to the Sensovation user manual to refocus imager and contact the PathogenDx Technical Team for further instruction.
Data cannot be generated	Arrays were scanned in the incorrect orientation	Check images and orientation of the array in the scanner and rescan.
DATA ANALYSIS TROUBLESHOOTING		
Unused wells have high probe signal	Unused wells or wells that have not come into contact with fluid will have higher background signal.	This is normal and the data from unused wells should be disregarded.
AUGURY ERROR CODES		
Error Code	**Possible cause**	**Resolution**
Corrupted Tiff File-Rescan Slide	One of the images in the slide was not properly scanned	A) Remove the .pdx file 2) Rescan the wells for the plate 3) Upload the newly scanned data
Red Channel Dim or Missing	PCR Inhibition	Refer to the PCR Trouble Shooting section

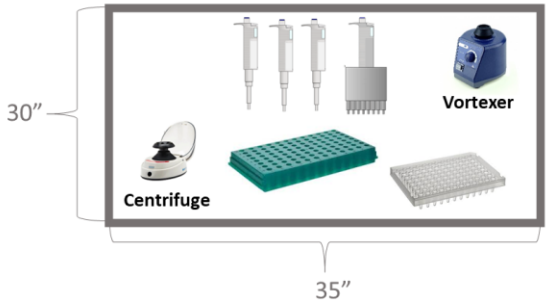
Issue observed	Possible cause	Resolution
	The wells may have come into contact with ethanol or isopropanol	Routine cleaning must be performed; however, user must ensure no carryover of cleaners including alcohols occurs. If the manifold has been cleaned allow copious amounts of MB grade water to pass through before performing a hybridization.
Slide Out of Focus	The Image is too blurry for the software to analyze	A) Rescan the 96 well plate ensuring the plate is flush with the tray and seated flat B) The imager may need to be refocused. Contact technical support for instructions on refocusing the imager.
Invalid Barcode	The barcode entered on the Image folder does not match a barcode in the PathogenDx software system	Review the barcode on the plate and change the name on the image folder with the correct barcode.
Bad Folder Name	Folder is not recognized by Augury	Carefully review the file name and update to the correct format
Slide or Slide Holder Placement Error	The plate or the slide holder is not flush in the scanner tray which is impacting the image	Rescan the 96 well plate ensuring proper placement and upload to the software for re-analysis. Make sure to delete the previously uploaded folders with the incorrect placement
Foreign Object in Well - Well xx	Debris is preventing proper imaging of the indicated well	Wipe the back of the 96 well plate with a lens paper or kimwipe. The chem wipe may be wetted with ethanol. Do not use a paper towel; lint if often the cause of this error.
	Lint on the back of the plate is preventing the software from analyzing the image	
Please Contact Technical Support	Unknown Issue Call	Call Tech Support at 1-800-641-2102 or send an email to techsupport@pathogendx.com
Slide Backward in Imager	The plate was imaged upside down or	Review placement ensuring the A1 position of the wells in the upper right hand corner of the scanner tray.
Arrays Too Close to Edge of Image	The plate or the slide holder is not flush in the scanner tray which is impacting the image	Rescan the 96 well plate ensuring proper placement and upload to the software for re-analysis. Make sure to delete the previously uploaded folders with the incorrect placement
Remove Dust from Slide and Re-Image	Lint on the back of the plate is preventing the software from analyzing the image	Wipe the back of the 96 well plate with a lens paper or kimwipe. The chem wipe may be wetted with ethanol. Do not use a paper towel; lint if often the cause of this error.
Slide Tilted in Imager	The plate or the slide holder is not flush in the scanner tray which is impacting the image	Rescan the 96 well plate ensuring proper placement and upload to the software for re-analysis. Make sure to delete the previously uploaded folders with the incorrect placement
Well not analyzed	User did not designate this well for analysis	If the well was intended by the user to be analyzed, resubmit the barcode with the correct well values.

APPENDIX A: LABORATORY SETUP GUIDELINES

Please refer to the following section for setting up your laboratory with PathogenDx specifications.

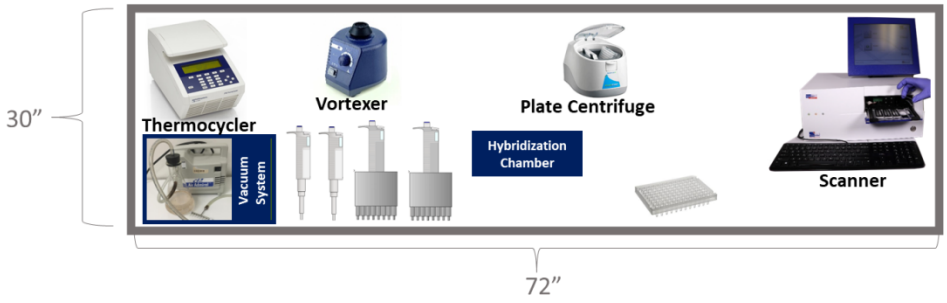
1. Always keep PCR reagents separate from areas where genomic DNA or the resulting post PCR product is used. This includes freezer space for enzymes, buffers, PCR Master mix, primers, positive control, taq etc.
2. PCR setup should be conducted in an area devoted **solely to PCR preparation**.
3. All supplies and instruments to be used for PCR preparation (water, pipettes, tips, tubes, plates, etc.) should be kept in the PCR PREP AREA and NEVER removed to areas where genomic DNA or amplicons are to be used.
4. Separate areas should be devoted to the use of genomic DNA and post-PCR product, respectively. All these workspaces may be housed in the same room, but materials from one space should NEVER be transferred to another to avoid contamination.
5. Equipment including pipettes, centrifuges, and vortex mixers MUST be dedicated equipment to each station. Proper cleaning and calibration must be performed.

PCR PREPARATION AREA: Required Square Footage is 7 sq. feet



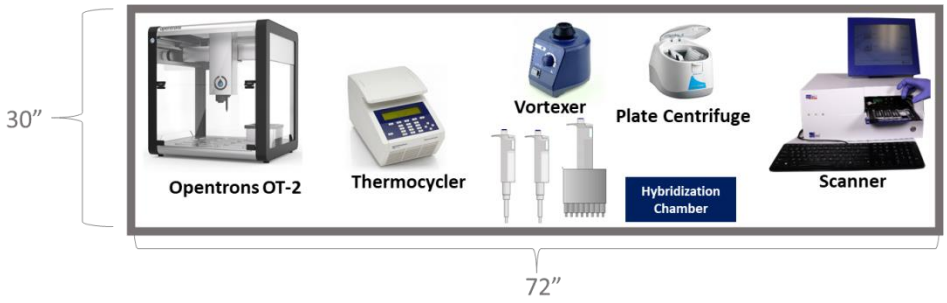
PathogenDx Kit Components	Equipment	Additional Materials
PCR Master Mix	Table top centrifuge: for quick spin of reagents	PCR thin-walled plates/tubes with seals/caps
		1.5mL and 5mL conical tubes and rack
Primer Set: ABR and Speciation		Pipettor: 2ul-20ul capacity & Barrier Tips
	Vortexer	Pipettor: 20ul-200ul capacity & Barrier Tips
Taq Polymerase		Pipettor: 200ul-1000ul capacity & Barrier Tips
		Multichannel pipettor: 10ul-100ul capacity

MANUAL HYBRIDIZATION/ POST PCR AREA Required Square Footage is 15 sq. ft



PathogenDx Kit Components	Equipment	Additional Materials
PathogenDx 96 well plate	Thermal Cycler	Molecular Biology Grade Water
	Centrifuge: PCR tubes/plates and Microarray Plates	1.5mL and 15mL conical tubes and racks
Buffer 1	Vortexer	Pipettor: 20ul-200ul capacity
	SensoSpot Microarray Analyzer (Imager)	Pipettor: 200ul-1000ul capacity
Buffer 2	Hybridization Chamber	Multichannel Pipettor: 30ul-300ul capacity
	Vacuum pump, flask, & multichannel aspirator setup with Stainless Steel 8 channel manifold	Multichannel Pipettor: 10ul-100ul capacity & Pipet Basins

AUTOMATED HYBRIDIZATION/ POST PCR AREA Required Square Footage is 15 sq. ft



PathogenDx Kit Components	Equipment	Additional Materials
PathogenDx 96 well plate	Thermal Cycler	Molecular Biology Grade Water
	Centrifuge: PCR tubes/plates and Microarray Plates	Troughs/Basins
Buffer 1	Vortexer	Pipettor: 20ul-200ul capacity
	SensoSpot Microarray Analyzer (Imager)	Pipettor: 200ul-1000ul capacity
Buffer 2	Hybridization Chamber	Multichannel Pipettor: 10ul-100ul capacity
	Opnetrons OT-2 Robot & Laptop	

APPENDIX B: MANUAL HYBRIDIZATION

Protocol for Manual Hybridization for labs running less than full columns of samples.

Equipment Required for Manual Hybridization

- Substitutions must be validated by the user.
- All equipment must be calibrated according to the manufacturer's specifications. It is the responsibility of the laboratory to maintain calibrated equipment.

Equipment	Number Required	Catalog #	Vendor
Pipettors		**Catalog #**	**Vendor**
Fisherbrand Elite Pipettors 20 to 200µL	1	FBE00200	Fisher Scientific
Fisherbrand Elite Pipettors 100 to 1000µL	1	FBE01000	Fisher Scientific
Fisherbrand Elite Multichannel Pipette 10 to 100µL	1	FBE800100	Fisher Scientific
Fisherbrand Elite Multichannel Pipette 30 to 300µL	1	FBE800300	Fisher Scientific
Aspiration System		**Catalog #**	**Vendor**
Argos Tech HandE-Vac Adapter 8 Channel Manifold	1	EW-04396-07	Cole Parmer
Welch Standard-Duty Vacuum Pump	1	01-055-60	Fisher Scientific
Flask and Tubing	1	15-0021	PathogenDx

Consumables Required (but not provided)

Part Description	Catalog #	Vendor
200µL barrier tips	1120-8810	USA Scientific
1000µL barrier tips	1126-7810	USA Scientific
5mL sterile tubes	4011-9487	USA Scientific
Disposable pipette basins 10mL	50-187-1120	Fisher Scientific
Disposable pipette basins 50mL	50-112-7246	Fisher Scientific
Kimwipes Delicate Task Wipers	06-666A	Fisher Scientific
Molecular Biology Grade Water– DNase/RNase Free	BP24701	Fisher Scientific
15mL polypropylene sterile tubes	12-565-268	Fisher Scientific
50mL conical polypropylene sterile tubes	12-565-270	Fisher Scientific

The materials listed above have been fully tested and are recommended for this platform. The use of alternative materials must be validated by the customer.

Hybridize PCR Amplified Product to the Microarray

General guidelines to follow for hybridization: When pipetting with the multichannel into the wells, only dispense to the first stop. Do not depress the multichannel to the second stop or fully evacuate the tips to avoid cross contamination.

When hybridizing a partial column, make sure the wells in the column that are not being used remain covered. Aspirate the partial column using a multichannel pipette by placing the pipette tips in a corner of the well and aspirate. Dispose into a waste container. Ensure all the liquid was aspirated from the well.

CAUTION: When pipetting with the (multichannel) pipette into the 96-well microarray plate, only dispense to the first stop. Do not depress the (multichannel) pipette to the second stop, or full evacuation of the tips, to avoid creating air bubbles which create aerosols resulting in cross contamination.

CAUTION: When pipetting with the (multichannel) pipette into the 96-well microarray plate, avoid contact of the tip(s) with the bottom of the well since the bottom of the well contains the printed microarray. Perform all actions using the (multichannel) pipette directed to the sidewall or corner of the well near the bottom.

CAUTION: Avoid contact with the entire bottom surface (outside glass plate) of the microarray 96-well plate during processing as this may adversely affect the clarity of the image acquisition. Use only the 96-well plate edges or barcoded area for handling.

Prepare the 96-well microarray plate for hybridization.

1. Perform all steps in the Hybridization/Post PCR Area.
2. Before starting, thaw Buffer 2 at room temperature.
3. Place the plate to be used in the Hybridization Chamber.
   a. Ensure the wells to be used have been clearly tracked.
   b. Carefully remove the foil seal from only the wells that will be hybridized. Use a clean razor blade or other precision blade to carefully cut the seal between the wells to be used and the wells that should remain covered for future use. Gently peel the seal from the wells you are going to use.
   c. Leave the remainder of the wells covered to avoid any contact with moisture.
4. Prepare the Pre-hybridization Buffer and Hybridization Buffers in sterile tubes for the number of wells that will be hybridized as per Tables 3 and 4. The tables shown below have the volumes required to make one well. Multiply the reagent volumes by the number of wells to be run. Add extra wells to account for pipetting loss. Vortex briefly to mix.
   a. Pre-hybridization, Hybridization, and Wash Buffers must be made fresh each time.

Table 3: Pre-Hybridization Buffer

Pre-hybridization Reagents	Volumes Corresponding to the Number of Wells Run												
	1 well	8 wells	16 wells	24 wells	32 wells	40 wells	48 wells	56 wells	64 wells	72 wells	80 wells	88 wells	96 wells
Molecular Biology Grade Water (µL)	137.6	1651	2752	3853	5229	6330	7430	8531	9907	11008	12109	13210	14310
Buffer 1 (µL)	40.9	490.8	818	1145	1554	1881	2209	2536	2945	3272	3599	3926	4254

Buffer 2 (µL)	21.5	258	430	602	817	989	1161	1333	1548	1720	1892	2064	2236

Table 4: Hybridization Buffer

Hybridization Reagents	Volumes Corresponding to the Number of Wells Run												
	1 well	8 wells	16 wells	24 wells	32 wells	40 wells	48 wells	56 wells	64 wells	72 wells	80 wells	88 wells	96 wells
Buffer 1 (µL)	11.8	141.6	236	330.4	448.4	542.8	637.2	731.6	849.6	944	1038	1133	1227
Buffer 2 (µL)	6.2	74.4	124	173.6	235.6	285.2	334.8	384.4	446.4	496	545.6	595.2	644.8

5. Apply 200µL of Molecular Biology Grade Water to each well while being careful to avoid contact with the microarray.
6. Aspirate and then again, dispense 200µL of Molecular Biology Grade Water to each well and allow to sit covered in the Hybridization Chamber for 5 minutes before aspirating water from the plate.
7. Aspirate the water wash and add 200µL of Pre-hybridization Buffer to each designated well of the PathogenDx plate without touching the pipette tip to the array surface. Close the Hybridization Chamber box lid.
8. Allow Pre-hybridization Buffer to stay on the arrays for 5 minutes; do not remove the plate from the Hybridization Chamber.

Hybridize the PCR amplified product to the microarrays.

CAUTION: During the following hybridization and wash steps, do not allow the microarray wells used for testing to air dry.

CAUTION: Avoid contacting the microarray surface with the pipette tip(s).

9. Briefly centrifuge the tubes or plate containing the PCR product.
10. Add **18µL** of Hybridization Buffer to each well of the Labeling PCR product for hybridization within the 96-well PCR plate or tubes, pipette up and down to mix. It is important that no cross-contamination occurs during this step. The PCR product and the Hybridization Buffer mix constitute the Hybridization Cocktail.
    a. Always dispense and evacuate in the well to avoid aerosolizing the PCR product or contaminating neighboring wells.
11. Aspirate the Pre-hybridization Cocktail from the arrays. **Caution:** Do not allow the arrays to air dry. Avoid contact with the array surface.
12. Immediately add 68µL (entire volume) of the Hybridization Cocktail to each array being careful not to touch the array surface with the pipette tip. Ensure that the sample ID and location are recorded.
13. Close the Hybridization Chamber lid.
14. Allow to hybridize for **2 hours** at room temperature in the Hybridization Chamber.

Post-Hybridization Wash of the Microarray

15. Prepare Wash Buffer according to the number of wells that were hybridized (Table 5). Multiply the reagent volumes by the number of wells to be run. Add extra wells to account for pipetting loss. Vortex briefly to mix. Washing must be performed according to the protocol to ensure detectable signal and adequate washing to prevent elevated background signals.
    a. Please note: The Buffer 1 volumes are listed in **µL** whereas the Molecular Biology Grade Water Volumes are listed in **mL.**

Table 5: Wash Buffer

Wash Buffer Reagents	Volumes Corresponding to the Number of Wells Run												
	1 well	**8 wells**	**16 wells**	**24 wells**	**32 wells**	**40 wells**	**48 wells**	**56 wells**	**64 wells**	**72 wells**	**80 wells**	**88 wells**	**96 wells**
Buffer 1 (µL)	**4.5**	54	90	126	171	207	243	279	324	360	396	432	468
Molecular Biology Grade Water (mL)	**0.5955**	**6.714**	**11.19**	**15.666**	**21.261**	**25.737**	**30.213**	**34.689**	**40.284**	**44.76**	**49.236**	**53.712**	**58.188**

16. Aspirate Hybridization Cocktail from the slides. Take care to avoid spillage to minimize lab contamination. This is a concentrated amplicon solution. Dispose of away from where PCR Preparation is performed.
17. Add 200µL of Wash Buffer to each array, then aspirate immediately.
18. Add 200µL of Wash Buffer a second time, close the Hybridization Chamber lid and allow buffer to remain on the slides for 10 minutes. Ensure 10 minutes have elapsed before aspirating. Control of this time is crucial as it will affect background and specificity.
19. Aspirate the Wash Buffer from step 4.
    Note: Steps 6-8 need to occur quickly to ensure the wash buffer does not dry on the array surface.
20. Perform a final wash by dispensing 200µL of Wash Buffer. Aspirate immediately.
21. Following the last aspiration step, remove the slides from the Hybridization Chamber.
22. Immediately after aspiration, dry the plate using the plate centrifuge for 1 minute.
    a. Place the plate face down with the open wells against paper towels to absorb liquid during centrifugation.
    b. After 1 minute, remove the plate and inspect for any remaining moisture. If moisture is present, repeat the centrifugation step until completely dry.
23. Prior to scanning, clean the back of the glass microarray with lens paper or Kim wipe (never use paper towels which leave an excess of fibers and interferes with scanning).
    a. If the back of the slide still shows dust and/or streaks, lightly spray a Kim wipe with 70% ethanol and wipe the back of the plate dry.
24. PathogenDx plates should be placed back into the moisture barrier bag with desiccant until scanning may be performed to protect the arrays from light. Once placed with desiccant in the dark, plates should be scanned within two weeks of hybridization.

PathogenDx

Thank you!

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NOT FOR USE FOR DIAGNOSTIC PROCEDURES.

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