savyonDIAGNOSTICS
member of the Gamida Diagnostics Division

NanoCHIP®

Clostridium difficile Panel

REF: 899061
Test kit for 192 determinations

Store at -20°C

For use with the NanoCHIP® 400 Instrument

For Professional Use Only IVD CE

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I. INTRODUCTION

Intended Use

The NanoCHIP® Clostridium Difficile Panel performed on the NanoCHIP® system is an automated qualitative *in vitro* diagnostic test for the direct detection the *Clostridium difficile* antigen and its toxins in human liquid or soft stool specimens from patients suspected of having *C. difficile* infection (CDI). The test is performed directly on extracted DNA from stool specimens and detects DNA gene markers of *C. difficile*. The test is intended to be used in the clinical laboratory in healthcare settings.

For *in-vitro* professional diagnostic use.

Background and Explanation of the Procedure

The gram-positive anaerobic bacillus *Clostridium difficile* is the leading causative agent of antibiotic-associated diarrhea and pseudomembranous colitis (1). This pathogen is capable of causing disease that could be severe or fatal if not diagnosed on time and treated. Exposure to antibiotics is the major risk factor for *C. difficile* infection. Infection can develop if the normal gastrointestinal flora is disrupted by antibiotic therapy and a person acquires toxin-producing *C. difficile*, typically via the fecal-oral route (2). *C. difficile's* key virulence factors are toxin A and toxin B (3, 4). These toxins show high sequence and functional homology. Toxin A has been described as a tissue damaging enterotoxin which attracts neutrophils and monocytes and toxin B as a potent cytotoxin that degrades the colonic epithelial cells (5). Most virulent strains produce both toxins, however, toxin A negative/toxin B positive strains are also capable of causing disease (6, 7). All strains of *C. difficile* produce high levels of GDH (8, 9). Therefore, *C. difficile's* GDH enzyme is considered a very good antigen marker for detection of this organism.

It is now evident that Hospital Acquired Infections (HAI) can be widely prevented through screening of patients before or during hospital admission and proper patient isolation and management. The NanoCHIP® Clostridium Difficile Panel is aiming to respond to this purpose as being a molecular-based diagnostic screening test for *C. difficile* directly from a variety of swab sample types. The detection relies on the identification of specific genes, known to be associated with the pathogen enzyme glutamate dehydrogenase (GDH) and its toxins A and B in stool samples. (Table 1).

The NanoCHIP® Clostridium Difficile Panel test is based on detecting genes that specifically mark the presence of the pathogen and its toxins in the sample. Following a multiplex Polymerase Chain Reaction (PCR) in a single tube, the resulted amplicons are hybridized to complementary capture oligonucleotides, which are embedded in a hydrogel layer that covers the microarray. The capture oligonucleotides are biotinylated at the 5' or 3' end, and are bound to streptavidin which has been previously incorporated in the hydrogel. The capture oligonucleotides as well as the amplicons are electronically addressed to specific loci upon their applications. Target identification is made by specific fluorescent reporter oligonucleotides that passively hybridize to complementary sequences on the amplicons. Sequential cycles of hybridization-imaging-thermal stripping of the fluorescent reporters allow the high multiplex capabilities of the system.

Table 1: Target genes used for identification of the bacteria in the Clostridium difficile Panel assay

Bacteria	Target gene
Clostridium difficile (+Toxins)	*gdh, tcdA, tcdB*

Principles of the Procedure

Specimen is collected and transported to the laboratory using the recommended swab (refer to "Equipment and Materials Required But Not Provided" section). The swab is then subjected to a short and simple procedure of DNA extraction by the user, alternatively the DNA can be purified from the swab by automation process. Once DNA is extracted, 2.5 µl of each sample is loaded into the PCR plate along with the PCR mix reagents for multiplex amplification in a thermo-cycler. As soon as the amplification process is completed, the PCR plate is loaded onto the NanoCHIP® 400 instrument along with the NanoCHIP® cartridge and the NanoCHIP® Clostridium Difficile Panel kit reagents, the run is started and no further operator intervention is required. The assay also includes a sample extraction control for each tested sample and one positive control to indicate that the whole process from DNA extraction throughout the NanoCHIP® reactions functions properly.

Kit Contents

The NanoCHIP® Clostridium Difficile Panel Kit contains enough purification, amplification and primer mix buffers for 192 samples/controls and enough detection reagents for four detection runs. One to 96 samples/controls can be analyzed in a single detection run. Refer to product package insert for performance characteristics and additional storage information.

Kit Box Storage

	≤ -20°C

Using NanoCHIP® Cartridge

The NanoCHIP® Clostridium Difficile Panel Kit is designed to analyze up to 192 samples on a NanoCHIP® 400 Cartridge. A cartridge may be used until the available test sites on the chip are utilized. Store either brand new or used cartridges at 2-8°C. The used cartridge may be reused up to 2 months if stored properly.

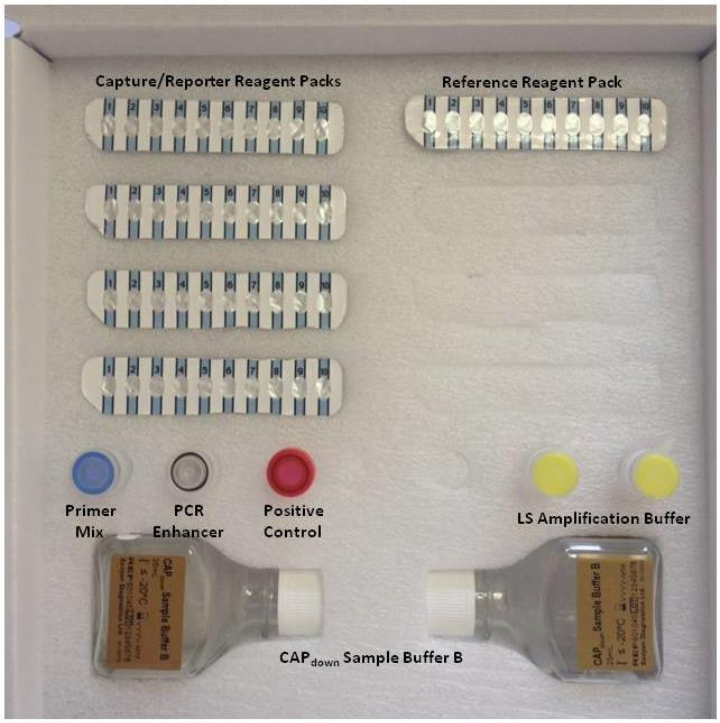
NanoCHIP® Cartridge Handling 

Handle the cartridge by holding the outer black covering only; do not touch the clear plastic or electrical contact area. Exposure to static electricity may damage the cartridge and may affect results. Ensure that the flowcell window (clear plastic on the underside of the cartridge) is clear of any debris. If debris is present, always use a new (not previously opened) Bausch & Lomb Pre-Moistened Tissue to clean the window. DO NOT use excessive force when wiping the flowcell window. Clean the flowcell window only if debris is present.

II. MATERIALS AND EQUIPMENT

Kit Reagents Contents

Product Description	Contents
Clostridium Difficile Panel Kit; *192 Samples*; Cat.# 899061 	4 x CDIFF Capture/Reporter Reagent Pack 1 x CDIFF Reference Reagent Pack 2 x vial (25 µL) PCR Enhancer 1 x vial (30 µL) Positive Control (red cap) 1 x vial (700 µL) CDIFF Primer Mix (blue cap) 2 x vial (850 µL) LS Amplification Buffer (yellow cap) 2 x CAP_{down} Sample Buffer B (25 ml)



Materials Available from Savyon

Ref.	Description	Contents
800160	NanoCHIP® 400 Cartridge	1 cartridge
800161	NanoCHIP® 400 Fluidics Cartridges	4 x fluidics cartridges
800154	NC400 Low Salt Buffer	6 x bottles (25 mL each)
800155	NC400 High Salt Buffer	6 x bottles (25 mL each)
800156	NC400 Target Prep Buffer	6 x bottles (25 mL each)
800061	NanoCHIP® Microplate Seals	100 x 96-well plate seals

Equipment and Materials Required But Not Provided

Plasticware and Consumables

- Sample Plates
  - 96-well ABI PCR plates (ABI N801-0560) or 96-well Thermo-Fast PCR plates (AB-1100)
- MicroAmp™ Compression Pads (ABI 4312639)
- 0.2 µm filters (Nalgene 5660020)
- Cotten swabs

Reagents

- FastStart Taq DNA polymerase (Roche) Cat# 04 738 420 001
- Reagents to run NanoCHIP® 400 system:
  - L-histidine (Sigma H-8000)
  - Triton® X-100 (Sigma X-100)
  - Water, deionized

Required Equipment

- NanoCHIP® 400 System
- A Thermal Cycler[1]

[1] *The following models are recommended:*
GeneAmp® Thermal Cycler 2700, 2720, or 9700
MJ Research Peltier Thermal Cycler PTC200
Biometra T Gradient Thermocycler

III. PRECAUTIONS

Amplification technologies can amplify target nucleic acid sequences over a billion-fold and provide a means of detecting very low concentrations of target. Care must be taken to avoid contamination of samples with target molecules from other samples, or amplicons from previous amplifications. Follow these recommendations to help control contamination.

1. Separate pre-amplification steps from post-amplification steps. Use separate locations for pre- and post-amplification. Use dedicated lab equipmentfor each stage. Prepare samples in a laminar flow hood using dedicated equipment to minimize contamination. Set up the post-amplification area in a low-traffic area with dedicated equipment.
2. Use disposable containers, disposable barrier pipette tips, disposable bench pads, and disposable gloves. Avoid washable lab wear.
3. Use a diluted bleach solution (0.2% sodium hypochlorite) to treat waste from the post-amplification and detection areas, as the waste contains amplicon. Use the bleach solution to wipe down equipment and bench areas, and to treat drains used to dispose of liquid waste.
4. Monitor contamination with regular swabbing. Use a wet cotton swab to wipe areas of the bench or equipment, and rinse the swab with 500 µL of water. Test a few microliters of the rinse solution in the amplification assay to detect possible contamination. If contamination is detected, follow internal de-contamination procedures.
5. Use negative controls to monitor for possible contamination during reaction setup. If reagent contamination is detected, dispose of the suspect reagents.

References for Contamination Control

- Kwok, S. and Higuchi, R. (1989). Avoiding false positives with PCR. Nature (London) 339, 237.
- Victor, T. et al. (1993). Laboratory experience and guidelines for avoiding false positive polymerase chain reaction results. Eur. J. Clin. Chem. Clin. Biochem. 31, 531.
- Yap, E.P.H. et al. (1994). False-positives and contamination in PCR. In: PCR Technology: Current Innovations. Griffin, H.G. and Griffin, A.M., eds., CRC Press, Boca Raton, FL.

IV. INSTRUCTIONS FOR USE

Sample Collection/Transport

In order to obtain an adequate sample, the procedure for sample collection must be followed closely. Using a dry, clean container, liquid or soft stool samples are collected and according to the following procedure.

Transfer liquid or soft stool (but not urine) into the container. Avoid mixing toilet paper, water or soap with the sample. Label the container. Ship the container to the laboratory according to the hospital standard operating procedures (Refer to ‘‘Storage and Stability’’ section).

Sample storage and stability

Collected specimens received in the laboratory should be processed upon arrival. In case of delay, store specimens refrigerated (2-8°C) for up to 72 hours or at -20°C for a longer period. Store purified nucleic acids at ≤ – 20°C. Protect against exposure to excessive heat.

Sample Preparation for PCR

DNA pretreatment manual Procedure

1. Prepare 2 ml screw cap tubes with 500 µl of **Extraction Buffer*.**
2. Open the sample container, dip and coat lightly (with swirl movements) a cotton swab with the sample.
3. Take out carefully the swab from the container into the 2ml buffer tube. Swirl the swab vigorously in the Extraction buffer and dispose the swab
4. Close the 2ml tube cap and Incubate at 97°C for 30 min.
5. Carefully remove the samples from the heater.
6. Take 2.5 µl sample for PCR amplification process.
7. Store crowd DNA at -20°C.

**** Extraction buffer is provided upon request.***

DNA Extraction Automate Procedure

DNA can be extracted from the sample's swabs using available commercial kits, according to the manufacture instructions.

Amplification

The following will be performed in an amplicon-free area.

1. Remove the LS Amplification Buffer and the CDIFF Primer Mix from the ≤ -20°C freezer. Thaw at room temperature and vortex.

2. Prepare PCR Master Mix using the following guidelines per sample (see Table 2). To ensure an adequate volume of Master Mix, take the number of reactions and add 2. Multiply the sum by the volume of each component shown in Table 2.
   Note: Remove the Taq DNA Polymerase from the freezer immediately prior to use, and return to the freezer promptly after use.

Table 2: PCR[1] Guidelines for preparing the Master Mix

Component	Volume per one reaction (µl)
LS Amplification Buffer	6.35
PCR Enhancer	0.15
CDIFF Primer Mix	3
Taq DNA Polymerase	0.5
Total Master Mix	10

3. Add 10 µl of the PCR Master Mix to reaction wells in the PCR plate.
4. Add 2.5 µl of the **Positive Control** into the first position of the samples in the PCR plate.
5. Add 2.5 µl of each sample's nucleic acid to individual reaction wells containing the Master Mix.
6. Add 2.5 µl of ultra-pure water for the Negative Control to the last well containing the Master Mix.
   Notes: Do not scale up an amplification reaction; always use 12.5 µl reaction volumes. The concentration of the template DNA should be at least 10 ng/µl.
7. Seal the PCR plate with a microplate seal and place it into a thermal cycler.
   Notes: Place the ABI MicroAmp Compression Pad over the sealed PCR 96-well plate and close the lid of the thermal cycler.
8. Program the thermal cycler using the parameters described in Table 3.

Table 3: Clostridium Difficile Panel Assay, Thermal Cycler Parameters

Temperature (°C)	Time	Number of Cycles
95	4 minutes	1
95	30 seconds	43
60	1 min	
72	45 seconds	
72	5 minutes	1
4	Hold	

9. Once cycling is completed, remove the PCR plate from the thermal cycler. The prepared plate may be stored at 2-8°C for up to one week, or at ≤ -20°C for up to six months.

To optimize workflow, you can begin other activities during sample amplification. For example, you can prepare the system and thaw reagents. During cartridge initialization, you can write the protocol and prepare the sample plate.

[1] Refer to Appendix C: Legal Notices, for PCR information.

Sample Plate Preparation

Sample dilution for the NanoCHIP® Clostridium Difficile Panel assay can be performed automatically by the NanoCHIP instrument or alternatively by manual procedure for additional use of the DNA sample. Please choose the **Onboard Sample Dilution** option (mark with arrow in Figure 1) or alternatively by performing a **Manual Sample Dilution**.

Onboard Sample Dilution

Remove the ABI MicroAmp™ Compression Pad from the ABI PCR plate covered with Microplate Seal, attach the sample plate to the PCR Plate base and insert into plate position 2 of the NanoCHIP® 400. Please see more information of this procedure at the Running the assay section.

Note: The Onboard Dilution Option can only be used with the ABI 96 well plate (ABI N801-0560) or the Thermo-Fast 96-well PCR plates (AB-1100) attached to the PCR Base Plate. Use of other plate types may cause damage to the instrument.

Manual Sample Dilution

1. Take out CAP_{down} Sample Buffer B from the freezer. Upon thawing, vortex the solution thoroughly until all precipitates are dissolved.
   Note: Once thawed, CAP_{down} Sample Buffer B can be stored at 2-8°C for up to one week. Do not refreeze.
2. For each individual amplification reaction, pipette 60 µL of CAP_{down} Sample Buffer B into one well of a 96-well plate.
3. Add 8 µL of each amplification reaction into a well containing CAP_{down} Sample Buffer B. Carefully pipette up and down to mix.
4. Cover plate with a Microplate Seal.

Avoid opening PCR plate to prevent contamination with Positive Amplicons in the laboratory

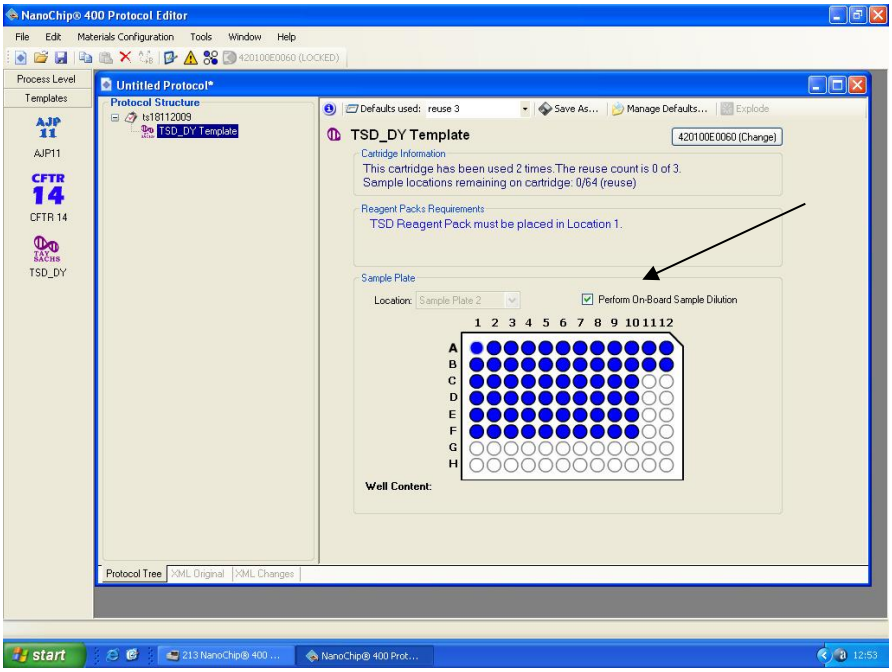


Figure 1. Protocol Editor Screen.

V. OPERATING THE NANOCHIP® 400 SYSTEM

Refer to the *NanoCHIP® 400 User's Guide (REF 140530)* for detailed instructions on the basic operation of the system, including system maintenance and cartridge handling.

Preparing Solutions for Use in the NanoCHIP® 400 Instrument

Preparing Wash Solution

It is required to prepare fresh Wash Solution (50 mM histidine, 0.1% Triton X-100) prior to daily run. Leftover of the solution should be disposed at the end of the day.

1. Add 500 mL of the 50 mM histidine solution to a 1L buffer bottle.
2. Add 2.5 mL of the 20% Triton X-100 solution and mix thoroughly.

50 mM histidine solution: In a bottle/beaker, add 7.8 g of L-histidine to a final volume of 1 L of dH_2O for 50 mM histidine. Mix until histidine powder is dissolved completely. Filter the solution using a 0.2 μm filter.
Note*:* This solution is stable for up to two weeks at 2–8ºC.

20% Triton X-100 solution: Add 4 mL or 4.24 g of Triton X-100 to approximately 15 mL of dH_2O for a final volume of 20 mL. Mix solution thoroughly (approximately 10 minutes).
Note: This solution is stable for up to three months at 2-8ºC.

Additional Solutions Required for the Run (Provided by Savyon)

The following table describes the required solutions, and their assigned locations within the instrument.

Table 4: Location of Bottles in the NanoCHIP® 400 Instrument

Solution	Bottle	Location	Minimum Volume*
Water	1 L	H_2O position	500 mL
Wash Solution	1 L	BUF position	500 mL
High Salt Buffer	30 mL	Position 1	25 mL
Low Salt Buffer	30 mL	Position 2	25 mL
Target Prep Buffer	30 mL	Position 3	25 mL
**CAP_{down} Sample Buffer B	30 mL	Position 4	25 mL

* The minimum volume of liquid that should be in the listed bottle before starting the assay run.
CAP_{down} Sample Buffer B is only required when performing **OnBoard dilution.

Preparing the NanoCHIP® Cartridge and Instrument

1. Take the following reagent packs out from the freezer and place them at room temperature to thaw.
   - CDIFF Capture/Reporter Reagent Pack
   - CDIFF Reference Reagent Pack

   The CDIFF Reference Reagent Pack is only required for the first use of a cartridge.

   Notes: These reagent packs must be used within 8 hours of thawing. All items listed above are single use only, discard after use.
2. Take a NanoCHIP® Cartridge out from 2-8°C storage. Keep at room temperature for at least 15 minutes before using.

   Note: Bringing the cartridge to room temperature before insertion into the instrument avoids the formation of condensation in the cartridge window, which could cause the cartridge to fail initialization.

3. Initialize and prime the NanoCHIP® 400 Instrument following the guidelines listed in the *NanoCHIP® 400 User's Guide.*
4. From the Dock Bar, select the instrument icon to start the NanoCH IP® 400 Instrument Manager
5. Ensure that the flowcell window (clear plastic on the underside of the cartridge) is clear of any debris. If debris is present, use a new (not previously opened) Bausch & Lomb Pre-Moistened Tissue to clean the window.
   Note: Do not use excessive force when wiping the flowcell window. Clean the flowcell only when debris is present.
6. Scan the barcode of the NanoCHIP® Cartridge using the attached barcode scanner.
   Note: The barcode will not be displayed in the Instrument Manager until step 8 has been completed.
7. Insert the cartridge into the instrument, ensuring that it is properly seated.
8. Close the cartridge door by pressing the button located below the cartridge slot on the instrument.
9. When the Cartridge Initialization window appears, select **Initialize Cartridge with Hydration** (see figure 2).

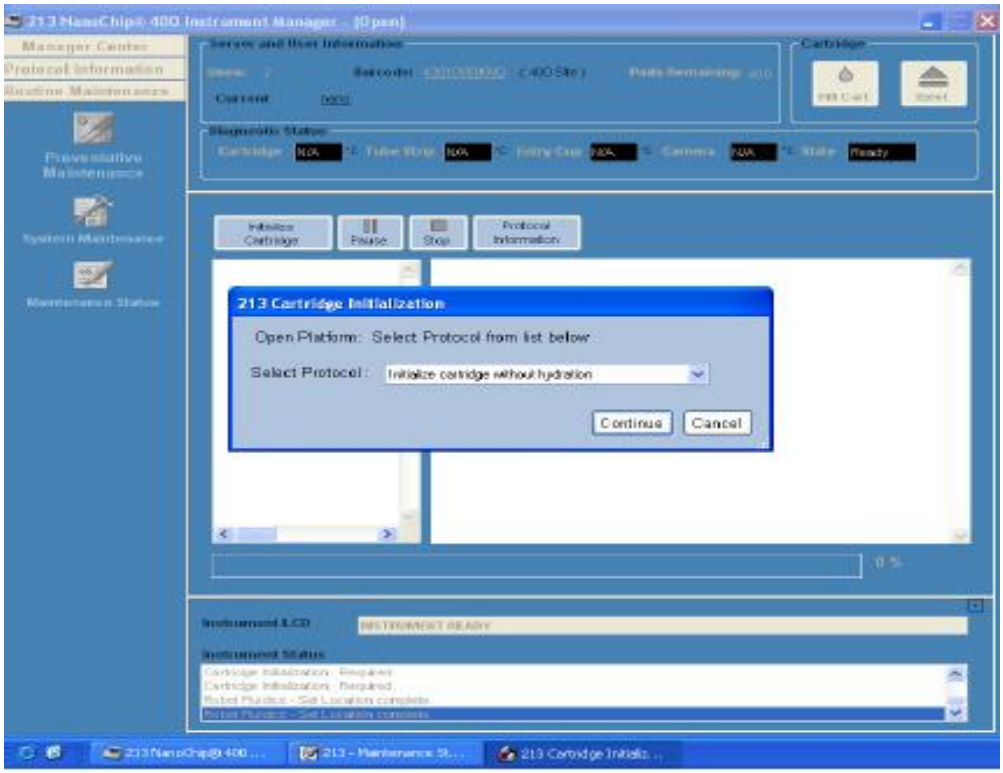


Figure 2: NC instrument Manager

10. Cartridge initialization will take approximately 18 minutes. When initialization is completed, the LCD will display "Instrument Ready".
11. Write the protocol as described in the following section.
    Note*:* The protocol can be written while the cartridge is initializing.

Creating a Protocol

Using the Protocol Editor, create the following protocol to address and report 1-96 samples. Create a new protocol for each sample run. For detailed instructions on using Protocol Editor, see the *NanoCHIP® 400 User's Guide*.

1. From the Dock Bar select **Protocol Editor** (see figure 3).

Figure 3. NanoChip 400 DockBar.

2. Select **Create A New Protocol**; select **OK**.
3. Select the **CDIFF** icon from the available templates on the left column.
   Note: The CDIFF template automatically determines prior pad utilization, and maps capture and sample addressing beginning with the first unused sample position.
4. The Plate Specification Window appears; choose the correct plate type intended for the assay from the options in the pull-down menu. Select OK.
   Note: Selecting a sample plate type other than what is placed on the NanoCHIP® 400 Instrument deck at the start of a run can cause damage to the system and fail the run. Use caution to select the appropriate plate type.
5. The Set Cartridge window appears; choose Select The Cartridge. From the pull-down menu, select the serial number of the cartridge that will be used in the run (or type the serial number into the window). Select **OK**.
   Note: If the cartridge selected is still initializing, a cartridge presently in use window will appear. Select **Yes** to indicate that you still want to use this cartridge for the protocol you are creating.
   Warning: Select **No** if the cartridge selected is in use with a Clostridium Difficile Panel Protocol and wait for the protocol to complete before creating a new Clostridium Difficile Panel protocol for the selected cartridge. If **Yes** is selected, the pad usage for the new protocol may not map correctly.
6. When a screen like the one shown in figure 4 appears, mark the wells with the samples on the plate drawing and select **"Perform On Board Dilution".**

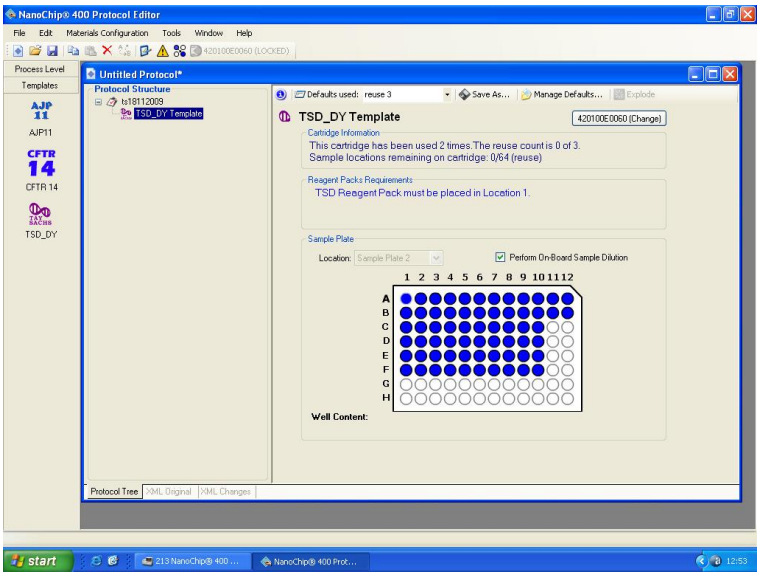


Figure 4. Protocol Editor Screen

7. Click on the run name you entered earlier (located under protocol structure), if no name was inserted it will appear as "untitled". **Scroll to 'Plate location 2'** (See figure 5). Here you can either write down your samples names or import it from an excel sheet. LIS connection is also optional.
   Note: A cartridge may be used up to 7 times for the CDIFF assay, or until the available test sites are utilized, whichever comes first.

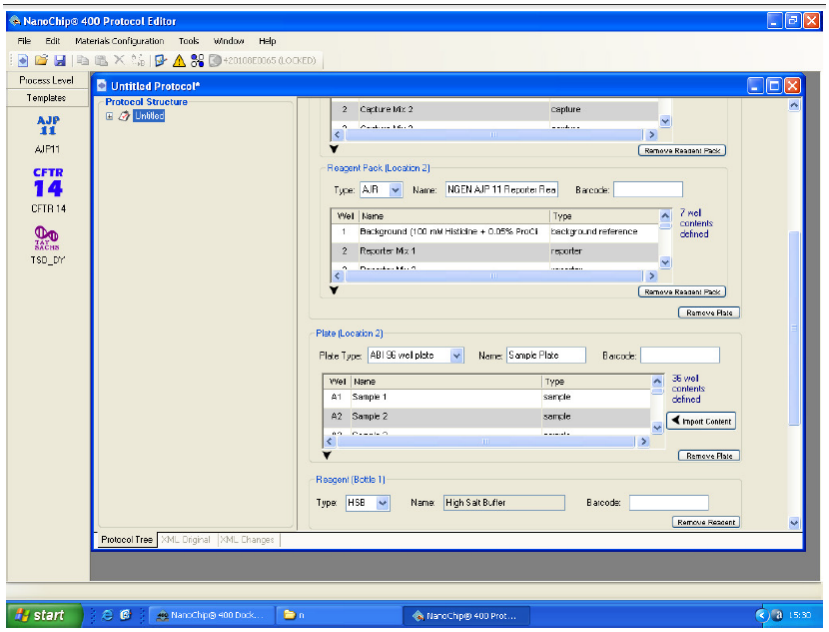


Figure 5. Protocol Editor Screen

8. **Click the template** (located on the left under protocol structure) click **'ok'** on the popup, this will make sure the template is updated with your recently added information. Make sure that on the plate drawing the wells you chose are blue with black dote in them.
9. Go to the Protocol Editor Toolbar and select the Protocol Summary icon  . A summary will display the materials configuration checklist that can be used when setting up the materials to run the protocol. To print out the summary, select the Print icon at the top left-hand side of the screen.
   Note: that the following details can be found in the Protocol Summary.
   - Estimated protocol run time.
   - Estimated waste volume to be generated.
   - Materials Configuration Checklist, including:
     - Water and Buffer Bottle contents and approximate volume consumption;
     - Reagent bottle contents and approximate volume consumption;
     - Reagent pack location and well contents;
     - Sample plate location with well contents and volumes.
   - Specific pad use.
10. Save the protocol by going to file "save as", click ok on the pad mapping popup and save the file under the proper library. Your protocol is now ready for run. Close the protocol editor

Running the Assay

1. Select the Instrument Manager icon from the DockBar to display an Instrument Manager screen. Selecting the Open button generates a browser allowing the user to select the desired protocol. When a protocol is selected the screen updates to display the details for that protocol
2. Ensure that the correct protocol is displayed before selecting the Run button. A pop-up showing the calculated volume of fluid waste that the protocol will be generates. If the waste container does not have enough room to hold the waste, empty the container and return it to its position under the

Instrument before selecting the OK button. After selecting the OK button, the user will be prompted to place the protocol materials in the Instrument.

3. Load reagents on the instrument deck
   a. Place the following buffer bottles on the instrument deck as instructed by the Instrument LCD prompts.

Table 5: Location of Bottles in the NanoCHIP® 400 Instrument

Solution	Bottle Size	Location
High Salt Buffer	30 mL	Slot 1
Low Salt Buffer	30 mL	Slot 2
Target Prep Buffer	30 mL	Slot 3
CAP_{down} Sample Buffer B*	30 mL	Slot 4

*Required for Onboard Sample Dilution option only. This position is left empty when sample dilution is done manually.

   b. Place the CDIFF Capture/Reporter Reagent Pack and the CDIFF Reference Pack Plate in the Reference Pack Plate before they are placed in the instrument deck as follows:
      - CDIFF Capture/Reporter– Position 1
      - CDIFF Reference Pack– Position 2
   c. Place the Reagent Pack Plate in the instrument deck (Location 1) as instructed by the LCD prompt.
4. Place the sample plate in Plate (Location 2) of the instrument deck as instructed by LCD prompt.
   Notes: When using an ABI 96-well sample plate on deck, always position the plate with well A1 in the upper left-hand corner.
5. Once the protocol materials are placed in the Instrument and the robot door is closed, the Instrument automatically runs the protocol. If the instrument needs to be stopped or paused, press the **Pause** or **Stop** buttons on the computer screen or the **Pause** button on the front of the Instrument. When the protocol has completed running, the Instrument LCD displays the message INSTRUMENT READY.
6. After the run is complete, select **Eject** from the Instrument Manager screen. When the LCD displays "Remove Cartridge", remove the cartridge from the instrument. If the cartridge has not been fully used, return the cartridge to its pouch and store at 2 - 8°C. If the cartridge has been fully used, discard it.
   Note: When the eject button is selected, a window will appear asking the user to strip and/or fill the cartridge before ejecting: Select **Fill;** Scroll down and Choose **Water**.
7. Remove all buffers and replace the Wash Buffer with water. Perform routine maintenance as is appropriate.

Results

The data are analyzed in a Microsoft Office Excel based spreadsheet. Refer to section IV for a description of the CDIFF Data Analysis Spreadsheet features, instructions for setting preferences, and data calculations.

1. Export the data from the NanoCHIP® 400 run as follows:

A. Select **Data Analysis** from the NanoCHIP® 400 DockBar
B. Select **Export Processed Data**. Select **Next**.
C. Select the appropriate cartridge and session number. The session numbers are listed by date, followed by the time the assay run started.
D. Select all red and green image data files; select **Finish**.
E. A new screen appears. In the View tab, select **Show Non-Activated Pads**.
F. Select **Export** on the lower right side of the NanoCHIP® 400 Data Analysis window.
G. A new screen will appear; be sure to check all the boxes and select **Export**.
H. Enter a file name (for example, the cartridge serial number and date of the run) and select **Save**. An Excel spreadsheet will automatically be generated.
I. Close the NanoCHIP® 400 Data Analysis software.

2. Import the CDIFF Panel data into the CDIFF Data Analysis Spreadsheet:
A. Open the CDIFF Data Analysis Spreadsheet.
B. Select the **Import** button. Find the file you just saved and select **Open**.
C. A new message appears that prompts the user to save the Data Analysis Spreadsheet. A default name is given, but another name may be assigned.
Notes: If Show Non-Activated Pads was not selected during data export, an error message will appear when data import is attempted to the CDIFF Panel Data Analysis Spreadsheet. If this occurs, repeat the data export process by selecting the Show Non-Activated Pads. To prevent data overwriting, the Import button is removed after a set of data is imported.
D. Select **Analyze** to view your results.
E. Save your changes to the spreadsheet

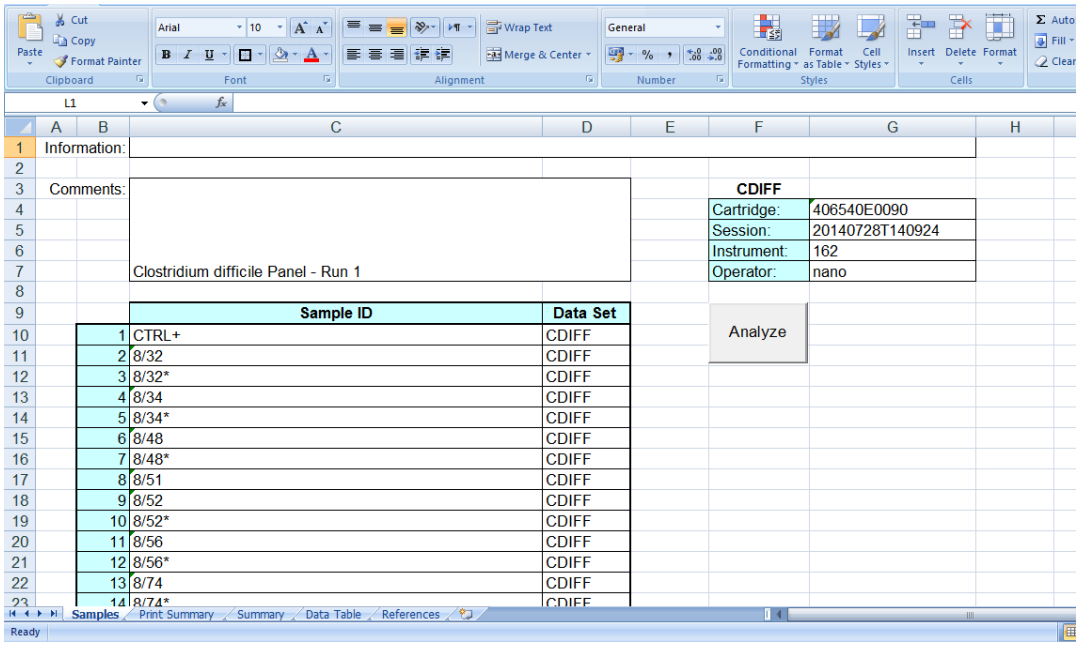
VI. CLOSTRIDIUM DIFFICILE PANEL DATA ANALYSIS

Clostridium Difficile Panel Worksheets

After importing and analyzing the test results into the "CDIFF Data Analysis Spreadsheet" (as explained in the Results section) the user can move between the different fields appearing in each sheet.

Samples Worksheet

The sample ID, cartridge number, cartridge session number, operator ID and instrument ID are imported to the Samples Worksheet. The Sample IDs and Sample ethnicities may be edited on this sheet. Boxes for the information header and comments are provided. All other cells are protected and cannot be edited. Footer with lines for “Reviewed By” and “Approved By” is on the printed sheet See figure 6.



Information:		
Comments:	Clostridium difficile Panel - Run 1	

CDIFF	
Cartridge:	406540E0090
Session:	20140728T140924
Instrument:	162
Operator:	nano

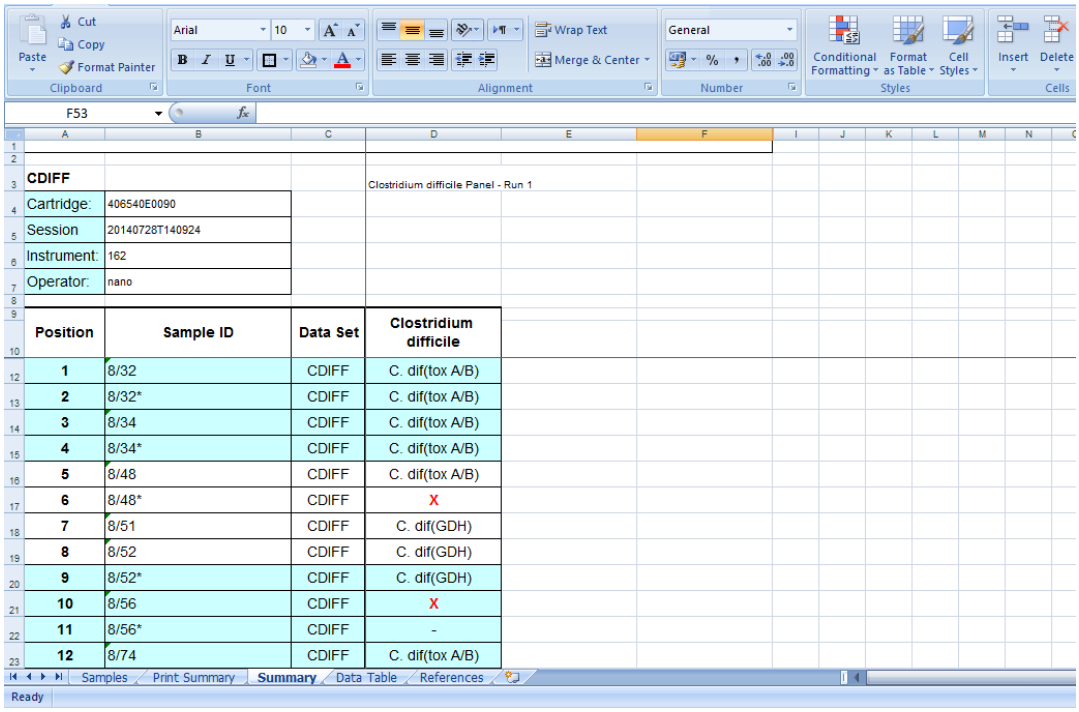
Analyze

	Sample ID	Data Set
1	CTRL+	CDIFF
2	8/32	CDIFF
3	8/32*	CDIFF
4	8/34	CDIFF
5	8/34*	CDIFF
6	8/48	CDIFF
7	8/48*	CDIFF
8	8/51	CDIFF
9	8/52	CDIFF
10	8/52*	CDIFF
11	8/56	CDIFF
12	8/56*	CDIFF
13	8/74	CDIFF
14	8/74*	CDIFF

Figure 6. CDIFF Data Analysis Spreadsheet; Samples worksheet.

Summary Worksheet

This sheet provides an overview of the sample calls. Sample positions that were run in the current session, sample IDs, Sample ethnicities, and results are displayed in adjacent columns as indicated in Figure 7. The interpretation relays on the presence of the fragments enhanced and detected in the reaction as mentioned in Table 1 and indicated in Table 6. The sample is Negative if all the markers are negative (-) and the Negative Validation Control (NVC) is positive. The sample is designated as "Invalid Sample" if all the target markers are negative (-). In this case there is a need for a new DNA extraction.).



CDIFF	
Cartridge:	406540E0090
Session	20140728T140924
Instrument:	162
Operator:	nano

Clostridium difficile Panel - Run 1

Position	Sample ID	Data Set	Clostridium difficile
1	8/32	CDIFF	C. dif(tox A/B)
2	8/32*	CDIFF	C. dif(tox A/B)
3	8/34	CDIFF	C. dif(tox A/B)
4	8/34*	CDIFF	C. dif(tox A/B)
5	8/48	CDIFF	C. dif(tox A/B)
6	8/48*	CDIFF	X
7	8/51	CDIFF	C. dif(GDH)
8	8/52	CDIFF	C. dif(GDH)
9	8/52*	CDIFF	C. dif(GDH)
10	8/56	CDIFF	X
11	8/56*	CDIFF	-
12	8/74	CDIFF	C. dif(tox A/B)

Figure 7. CDIFF Data Analysis Spreadsheet; Summary worksheet

The Summary Worksheet also displays the information header, cartridge number, cartridge session number, and operator ID. When printed, a footer with lines for “Reviewed By” and "Approved By” will appear. The print settings for this sheet are editable. All cells in this sheet are protected and cannot be edited

.

Table 6: Data Interpretation by Target Gene for Clostridium Difficile Panel

NVC	*gdh*	*tcdB*	*tcdA*	Interpretation
-/+	-	-	+	***No organism found***
-/+	-	+	-	***C. diff* (toxA/B)**
-/+	-	+	+	***C. diff* (toxA/B)**
-/+	+	+	+	***C. diff* (toxA/B)**
-/+	+	-	+	***C. diff* (toxA/B)**
-/+	+	+	-	***C. diff* (toxA/B)**
-/+	+	-	-	***C. diff* (GDH)**
+	-	-	-	***No organism found***
-	-	-	-	**Invalid sample**

Data Table Worksheet

The information displayed in the Data Table sheet are sample ID, cartridge number, cartridge session number, operator ID, and the calculated data that are described below. The information displayed for each sample and mix is the Green signal.

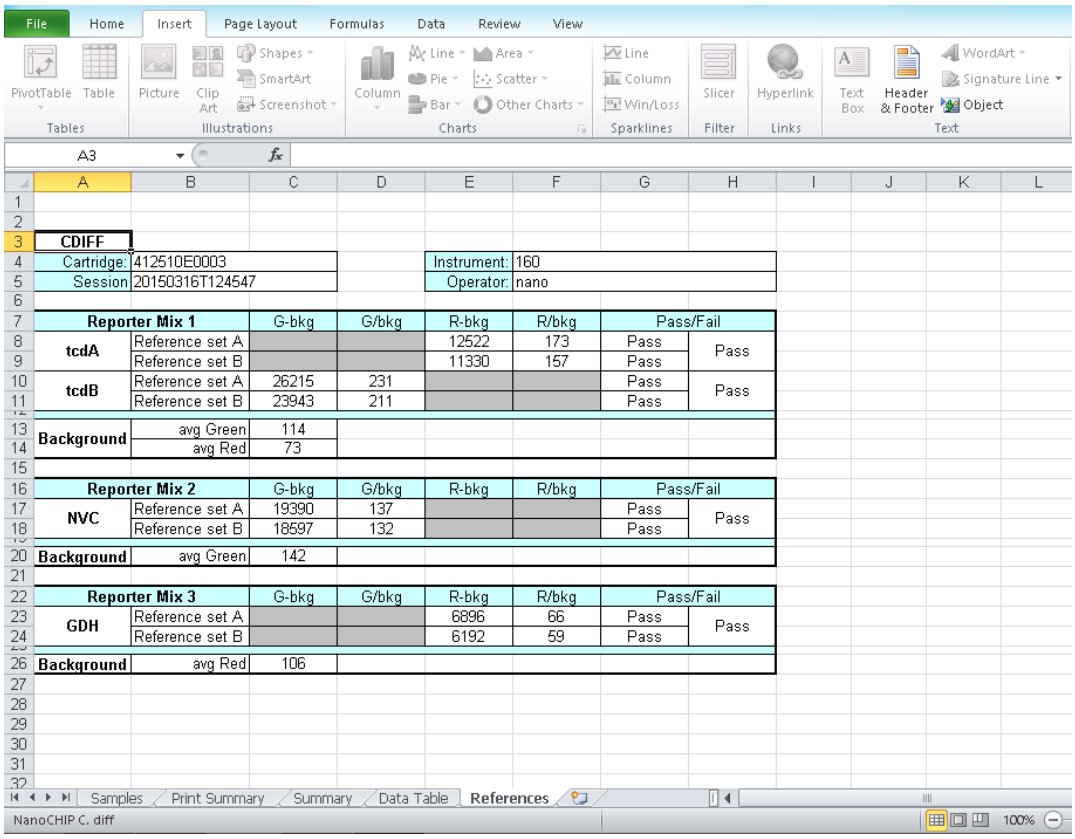
The Data Table Worksheet also displays the information header, cartridge number, cartridge session number, and operator ID. When printed, a footer with lines for “Reviewed By” and “Approved By” will appear. The print settings for this worksheet are editable. All cells in this sheet are protected and cannot be edited.

The Positive Control sample is used for the validation of the PCR. The call designated for the Positive Control is "Pass" as appearing in position 0 of the table.

References Worksheet

The signal data for the References and average Reference Mix backgrounds are listed on this sheet (Figure 8). Additionally, it lists whether or not the references pass the signal threshold and signal-to-background criteria. In the event that a Reference fails, all samples are designated as “Reference Failure” and no calls can be made on the Summary Worksheet or Data Table Worksheet. In this case, please refer to Table 8 below for further actions.

The References Worksheet also displays the information header, cartridge number, cartridge session number, and operator ID. When printed, footer with lines for “Reviewed By” and “Approved By” will appear. The print settings for this worksheet are editable. All cells in this sheet are protected and cannot be edited.



	CDIFF							
	Cartridge:	412510E0003			Instrument:	160		
	Session	20150316T124547			Operator:	nano		
	Reporter Mix 1		G-bkg	G/bkg	R-bkg	R/bkg	Pass/Fail	
	tcdA	Reference set A			12522	173	Pass	Pass
		Reference set B			11330	157	Pass	
	tcdB	Reference set A	26215	231			Pass	Pass
		Reference set B	23943	211			Pass	
	Background	avg Green	114					
		avg Red	73					
	Reporter Mix 2		G-bkg	G/bkg	R-bkg	R/bkg	Pass/Fail	
	NVC	Reference set A	19390	137			Pass	Pass
		Reference set B	18597	132			Pass	
	Background	avg Green	142					
	Reporter Mix 3		G-bkg	G/bkg	R-bkg	R/bkg	Pass/Fail	
	GDH	Reference set A			6896	66	Pass	Pass
		Reference set B			6192	59	Pass	
	Background	avg Red	106					

Figure 8. Clostridium Difficile Panel Data Analysis Spreadsheet; Reference worksheet

Data Calculations

Data calculation and analysis are performed automatically by the Excel software. The raw data is available for the user convenience.

In order to determine if a specific amplicon is present in the tested sample, the signal corresponding to this sample is compared to the signal from the irrelevant capture (background). The Flowchart in figure 9 describe the NanoCHIP Clostridium Difficile Panel application algorithm for determine if a certain sample is positive or negative

In viewing the Data Table Worksheet, the first column indicates the sample position on the cartridge. The sample column lists the sample ID. The marker column lists the markers present in the mix. Each row of markers for a sample corresponds to capture mixes 1 and 2 in order. The Green listed is the raw signals for that pad. The Control signal is the sample specific background. The ratio listed is the value of the Green signal of the sample divided by the Control signal of the same sample. This value determines the signal requirements and ratio criteria described in Table 7. For example, if a certain sample presenting signal above 4000 and ratio >4 for *gdh* reporter then the sample is indicated as positive for *gdh* antigen, namely presence of *Clostridium difficile*. If for the same sample, toxin A and/or B markers (reporters) are also positive according to the cut-off, the call will be designated as " C. difficile (tox A/B), i.e., toxigenic strain of *Clostridium difficile.*

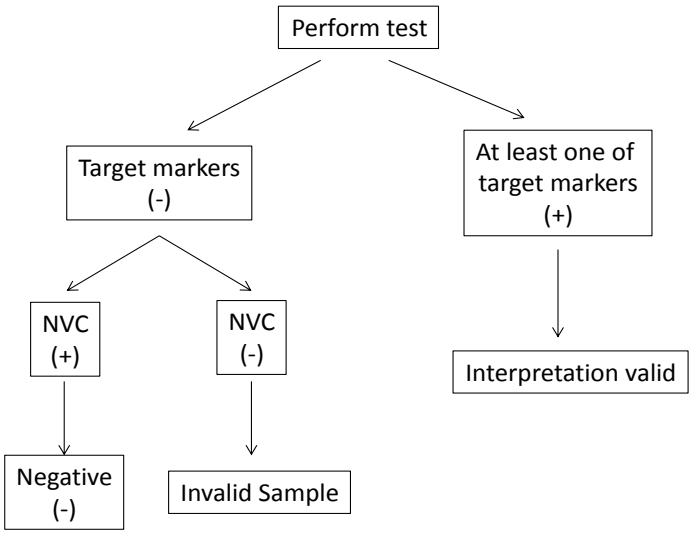


Figure 9. Data interpretation flowchart.

Table 7: Signal level and Ratio (S/N) of the C. difficile markers in the test.

Target	Minimum Signal	Minimum Ratio
C. difficile (tcdB)	5000	4
C. difficile (tcdA)	4000	4
C. difficile (gdh)	4000	4
NVC	5000	2

Troubleshooting

When the following observations of 1) Invalid sample 2) False positive in the negative control well or 3) Reference failure are noted in the Summary Worksheet of the assay, please follow the recommended solution.

Table 8: Troubleshooting

OBSERVATION	ACTION	PROBABALE CAUSE	SOLUTION
Specific samples are invalid		Nucleic acid extraction failure	Re-extract all invalid samples
PC failed and/ or all clinical samples are invalid	Check PC and NVC target's in the Data Table sheet	Specific problem of the PC sample or failed extraction process	Re-extract all samples tested in case and NVC signal reading is negative for all samples
False positive signal observed at the Negative control sample		Presence of contamination	Apply decontamination control plan. Repeat PCR amplification after decontaminating the work area
Reference failure		Improper NanoCHIP® procedure- user error	Repeat the NanoCHIP procedure according to manual

Limitations of the Procedure

1. The NanoCHIP® assays can only be performed only on the NanoCHIP® instruments.
2. The NanoCHIP® assay is a qualitative test and does not provide the quantitative value of detected organism present.
3. The NanoCHIP® Clostridium Difficile panel assay is intended for use only with cotton swab.
4. Positive results obtained using the NanoCHIP® Clostridium Difficile panel assay are presumptive. All results should be used and interpreted in the context of a full clinical evaluation as an aid in the diagnosis of gastrointestinal infection.
5. Error results may occur from improper sample collection, handling, storage, technical error, sample mix-up, or because the number of organisms in the sample is below the analytical sensitivity of the test.
6. As with all PCR-based in vitro diagnostic tests, extremely low levels of target below the analytical sensitivity of the assay may be detected, but results may not be reproducible.
7. If a certain sample result is Invalid then the sample should be repeated from DNA extraction.
8. NanoCHIP® failed Positive Control sample indicate of a problem in the amplification process and require new PCR for all samples in the same run.
9. As with all in vitro diagnostic tests, positive and negative predictive values are highly dependent on prevalence. The NanoCHIP® Clostridium Difficile panel performance may vary depending on the prevalence and population tested.

VII. PERFORMANCE CHARACTERISTICS

Clinical Sensitivity and Specificity

Clinical performance characteristics of the NanoCHIP® Clostridium Difficile Panel were assessed in a clinical study performed in external site by evaluation of clinically-obtained retrospective (frozen) specimens. Study specimens consisted of unpreserved samples (parasites testing) or Cary-Blair preserved (bacteria testing) liquid to soft stools from symptomatic patients with acute gastroenteritis for whom diagnostic procedures were indicated and ordered.

Specimens were characterized by the source site routine laboratory methodologies and included direct culture, microscopy or immunoassay which comprised the reference method for specimens obtained from the site. Genomic DNA was extracted using NucliSENS easyMag (bioMerieux) DNA extraction automated systems, as well as with manual stool DNA extraction kit (Qiagen). All extracts were stored at -20˚C before and after use.

Table 9, presents results obtained from retrospective evaluation performed in UK in comparison to the lab reference methods

Table 9: sensitivity and specificity of the NanoCHIP® Clostridium difficile panel assay from a retrospective study.

Pathogen	External evaluation			
	Positive Agreement		Negative Agreement	
	TP/ (TP+FN)	Percent	TN/ (TN+FP)	Percent
C. difficile(tcdA, tcdB)	84/84	100	998/1001	99.7
C. difficile(gdh)	99/99	100	555/594*	93.3*

* GIP combi I detected 139 *C. difficile*-gdh positive samples, whereas only 57 detected by laboratory routine. Forty two samples were confirmed by reference method. Further 40 identified as *C. difficile* (gdh) by GIP combi I, could not be confirmed since there was no target for gdh in the reference kit used during this evaluation.

APPENDICES

Appendix A: Clostridium Difficile Panel Format

The Clostridium Difficile Panel assay uses a capture down format to recognize the markers based on identified sample ethnicity. Following the single tube multiplex polymerase chain reaction, the amplicons are specifically bound to a permeation layer that covers the electronic microarray via hybridization to complementary capture oligonucleotides. These captured oligonucleotides are biotinylated at the 5' or 3' end and are bound to streptavidin that has been incorporated into the permeation layer.

The Clostridium Difficile Panel Kit components include the following:

1. CDIFF Primer Mix: set of forward and reverse amplification primers that specifically amplify fragments (markers) that are shown to be associated or conferring resistance to antibiotic agents (Table 1).
2. LS Amplification Buffer: a general purpose reagent used for the PCR amplification of DNA in an ionic environment optimized for analysis on the NanoCHIP®400 electronic microarray.
3. CDIFF Capture/Reporter Pack: the pack containing a set of 2 unique capture mixes and 3 unique reporter mixes. Each capture is a biotinylated synthetic oligonucleotide complementary to one of the amplicons generated with the CDIFF Primer Mix. Each capture is present in one of the 2 capture mixes. Reporter mixes contain discriminators and universal reporters. Each discriminator contains a segment that is complementary to the fragments that specifically characterize the pathogens of interest. Each Clostridium Difficile reporter mix contains numerous pairs of discriminators.
4. CDIFF Reference Pack: the pack containing a set of 2 unique mixes of biotinylated reference oligonucleotides. The reference oligonucleotides have a segment complementary to one or more discriminator oligonucleotides. The green/red signals generated from the references indicate that the reporter mixes and reporting protocol are working properly.
5. CAP_{down} Sample Buffer B: a general purpose reagent used for the delivery of amplicons to the activated test sites on the NanoCHIP®400 electronic microarray.

Starting with the amplified material, the Clostridium Difficile Panel protocols generated as described in the "Creating a Protocol" section consist of the following five steps.

1. **Capture addressing:** the capture oligonucleotide mixes specific for the Clostridium Difficile Panel assay are electronically addressed to predetermined pads across the cartridge in a sequential manner. The number of pads addressed with each mix is equal to the number of samples/controls being analyzed. Wells 1 and 2 of the CDIFF Capture/Reporter Reagent Pack contain Capture Mixes 1 and 2.
2. **Reference addressing:** the reference oligonucleotide mixes specific for the Clostridium Difficile Panel assay are electronically addressed to predetermined pads in the NanoCHIP® microarray. Each reference mix is addressed in two separate electronic activation events to separate pads. References are addressed in the first use of the cartridge subsequent cartridge runs utilize references addressed in the first use. The reference mixes are in wells 1 and 2 of the Clostridium Difficile Panel Reference Reagent Pack.
3. **Amplicon Hybridization:** amplification reaction products diluted in CAP_{down} Sample Buffer B are simultaneously addressed to 2 pads that comprise the full set of the Capture Mixes 1 and 2. The amplicons are sorted across the 2 pads by hybridization to specific captures. An amplicon hybridizes only 1 of the 2 capture pads.
4. **Reporting:** sequential cycles of passive hybridization-thermal discrimination-fluorescence imaging-thermal stripping ensure for each of the 3 reporter mixes contained in the CDIFF Capture/Reporter Reagent Pack in wells 3-5. The thermal stripping step removes the discriminator/universal reporters but leaves the amplicon bound to the capture oligonucleotide for the next reporter mix.

5. **Reverse Bias Washing:** each pad that was addressed with sample is subjected to a reverse bias wash to remove bound amplicon that can potentially interfere with future assays on the microarray. After Reverse Bias Washing, the system automatically fills the cartridge with Water for storage between uses.

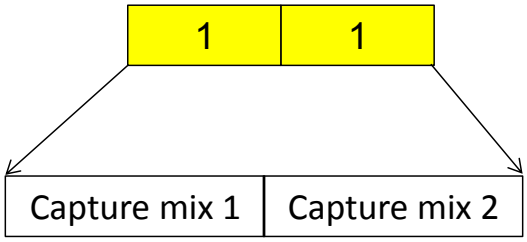


Figure 10: Map of capture Mix Pads arrangement

Table 12 displays the markers in the context of the capture/reporter mixture matrix. Each reporter mix reports markers across the 2 sample pads and has a minimum of one pad not used. The unused pad serves as the background for that reporting. Each sample has its own background pad.

Table 12: Map of Reporter Mixes 1–3 across Capture Pads 1–2

	Reporter Mix 1		Reporter Mix 2	Reporter Mix 3
	RED	**GREEN**	**GREEN**	**RED**
Capture Mix 1	*tcdA*	*tcdB*	Control	*gdh*
Capture Mix 2	Control	Control	NVC	Control

Appendix B: Legal Notices

Notice to Recipients about Licenses

Certain usages of the product described herein have been licensed from Beckman-Coulter Incorporated under United States Patent No. 5,653,939 and foreign counterparts thereof, and may be covered by Genetic Technologies Limited, United States Patent No. 5,612,179, applications and foreign counterparts thereof.

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