# Molecular detection of *Clostridium difficile*

HGCDIFFR

# Instructions for Use

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CE

# 1. Kit Contents

HG *C. difficile* 30-test kits contain:

| | Description |
|---|---|
| **HG *C difficile* Reaction Strips**<br><br>**Part # CDIFF-RX** | 3 pouches, each containing 10 x 2-tube reaction strips with desiccant sachets<br><br>Reaction strips contain complete lyophilized reaction mixes for *Clostridium difficile* and Extraction Control |
| **HG *C difficile* Dilution Buffer 1 (Green caps)**<br><br>**Part # CDIFF-DB1** | 30 x vials of Dilution Buffer 1 (1.5ml), each containing Extraction Control plasmid with preservative. |
| **HG *C difficile* Dilution Buffer 2 (Yellow caps)**<br><br>**Part # CDIFF-DB2** | 30 x vials of Dilution Buffer 2 (0.7ml), each containing Extraction Control plasmid with preservative. |
| **Swabs** | 30 x dry swabs for sampling stool from primary collection vessel. |

# 2. Shelf life & Storage

- Kits must be stored at 2-8°C and used before the expiry date on the kit label.
- Reaction strip pouches must be re-sealed fully after use and returned to storage at 2-8°C.
- Opened vials of Dilution Buffer should be discarded after use.
- All 10 reaction strips from an individual pouch must be used within 28 days of initial opening of the pouch.

# 3. Accessories

**Supplied by HiberGene:**

- **HG Swift Strip Carriers** are supplied with the HG Swift instrument and are used for handling, loading and unloading of HG *C. difficile* reaction strips. These are also available to order separately (Part # HGCAR)
- **HG Swift Set-up Racks** are supplied with the HG Swift instrument and are used for loading samples into HG *C. difficile* reaction strips. These are also available to order separately (Part # HGRACK)
- **HG *C. difficile* Control** (Part # HGCDIFFC) is a positive control available as a separate kit from HiberGene. This provides a control for the performance of both Target and Extraction Control reaction mixes.

**Required but not provided:**

- Laboratory scissors
- Dry bath/Heat block – the selected thermal block must be capable of holding 2ml tubes and maintaining a temperature of 105°C with an accuracy of +/-1°C.
- Calibrated micropipettes.
- Micropipette tips with filters, certified nuclease-free.
- Centrifuge – the selected centrifuge must be capable of holding 2.0ml microcentrifuge tubes and HiberGene reaction strips and of spinning at 2700-3300 rcf.
- Vortex mixer.

# 4. Intended Use

HG *C. difficile* is a LAMP-based molecular diagnostic test for the detection of *Clostridium difficile* in unformed human stool samples.

The intended end user is a trained laboratory/health professional. Users must have received training from the distributor/Hibergene staff prior to using the device.

# 5. *Clostridium difficile* – Clinical Significance

*Clostridium difficile* (*C. difficile*) is a gram-positive spore-forming bacterium which is a major cause of nosocomial diarrhoea. *C. difficile* colonization of the gut can occur when antibiotic exposure alters the natural composition of the intestinal flora, leading to *Clostridium difficile* infection (CDI).

Although *C. difficile* may be present in the gut of up to 7 - 15% of healthy adults [1], treatment with antibiotics can result in survival of bacterial spores and subsequent overgrowth of the organism. Toxigenic strains of *C. difficile* release a number of toxins – primarily toxin A and toxin B – which can damage the mucosa, causing colitis and diarrhoea. Up to 25% of antibiotic-associated diarrhoea (AAD) is caused by *C. difficile* infection [2], which may be fatal, particularly in elderly patients, with one study reporting mortality rates ≥ 20% in patients older than 65 years of age during an epidemic [3].

*C. difficile* is transmitted via the faecal-oral route, and – as spores are highly resistant to standard cleaning methods in clinical environments – the pathogen can spread rapidly to cause outbreaks in healthcare facilities if undetected.

Numerous strains of *C. difficile* have been isolated, and techniques including toxinotyping and ribotyping have been used to characterize and classify pathogenic strains. A range of hypervirulent variants, collectively classified as NAPI/B1/027 strains, which began to emerge and cause epidemics in 2000, exhibit increased resistance to fluoroquinolone antibiotics [4].

All pathogenic strains of *C. difficile* carry genes for toxins A and B (*tcdA* and *tcdB*, respectively); although a number of toxinotypes have been identified which are toxin B-positive, toxin A-negative and which contain deletions at the 3' end of *tcdA*, the 5' end of the gene is intact in these strains [5].

Although toxigenic culture is considered by many to be the reference method for detection of *C. difficile*, this time-consuming method was later superseded by toxin enzyme immunoassays (EIAs) for diagnosis. The enzyme glutamate dehydrogenase (GDH) is produced at high levels by both toxigenic and non-toxigenic strains of *C. difficile*, and immunoassays detecting GDH are often used to rule out CDI prior to further testing. More recently, nucleic acid amplification tests have been shown to demonstrate much-improved sensitivity and turnaround time relative to older methods [6].

## 6. Principle of the Assay

The HG *C. difficile* assay utilizes isothermal LAMP technology to deliver a rapid and sensitive testing solution for *C. difficile*, with an integrated sample handling control. HiberGene's embodiment of LAMP technology (loop-mediated isothermal amplification [7,8] employs novel primer design, a highly efficient strand-displacing polymerase and an intercalating dye to facilitate specific fluorescent detection at constant temperature.

A LAMP assay targeting a region of the *tcdA* gene conserved in both toxin A+/B+ and toxin A-/B+ strains of *C. difficile* was designed, and LAMP primers for this region are contained in the *C. difficile* Target reaction mix. Also present in the reaction strip are the primers for a bacteriophage sequence, which acts as the Extraction Control (EC) to demonstrate recovery of DNA from the primary sample, the absence of inhibitors, and to control that all the stages of the assay have worked correctly.

Both Target and EC reaction mixes are presented in a ready-to-use lyophilized format, containing the primers, enzyme, cofactor and buffering components required to allow LAMP amplification when reconstituted with the sample or extract.

The reaction mixes also include intercalating dye which increase in fluorescence as double-stranded LAMP amplification product is formed. This increase in fluorescence is monitored in real-time on board the HG Swift instrument, which also maintains precise temperature control for the duration of the run.

# 7. Precautions

**General Precautions**

- The HG *C. difficile* kit is for *in vitro* diagnostic use only.
- *Training on the test protocol must be carried out before use of the test.*
- Appropriate personal protective equipment should be worn when handling clinical specimens. Gloves should be worn at all times during sample handling and assay-set-up.
- Specimens should be handled and disposed of per Good Laboratory Practice, and all specimens should be dealt with as if infectious. Hands should be washed thoroughly after handling clinical specimens.
- Surplus kit components should be disposed of in accordance with establish safety procedures.
- Never mouth-pipette, eat or drink in the laboratory.
- To avoid burns, exercise caution when removing samples from 105°C incubation.
- Take every care to avoid cross-contamination between samples during extraction and assay set-up.
- Only HG *C. difficile* Reaction Mix and Dilution Buffers from the same kit lot should be used together.
- Pipette tips used should include filters and be certified nuclease-free. Micropipettes used should be calibrated in accordance with applicable guidelines.
- Clean down all work surfaces after assay runs with a disinfectant solution with proven efficacy in DNA removal.
  *Recommended cleaning solution: Invitrogen™, DNAZap™ PCR DNA Degradation Solutions or similar.*
- The instrument should not be used in an area with a high or low magnetic field.
- Kits with damaged packaging or opened pouches should not be used.

**Preventing amplicon contamination**

Escape of amplified material from the reaction strips after LAMP amplification can result in laboratory contamination which could impact on subsequent test results. HG *C. difficile* reaction strips are specifically designed to resist accidental reopening, but the following specific precautions **must always** be followed:

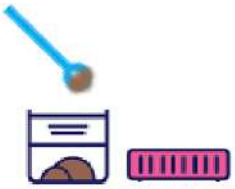
- After sample is added into tubes, close caps **firmly and completely**.
- **Never re-open** the caps of the reaction strips after closing.
- After the run, remove the reaction tube strip from the HG Swift, lifting by the handles of the Strip Carrier.
- Dispose of the used strips firstly into a small sealable plastic bag and then into a bin. Empty the bin regularly and do not allow large amounts of waste to build up on top of bags containing used reaction strips.
- Work areas must be regularly cleaned with appropriate DNA decontamination solutions.
- It is recommended to run NTCs periodically to check for contamination.

## 8. Performing the Test

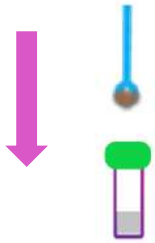
The flowchart below summarizes the workflow to be followed for testing of stool samples:

1. **Collect stool sample according to clinical guidelines.**

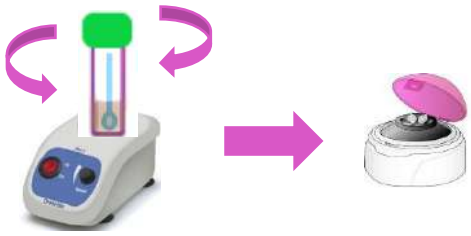
2. **Swab the stool sample by covering 75% of the swab**



3. **Add the swap to Dilution Buffer 1 (green lid). Dip up and down 10 times and leave the swab in the tube.**

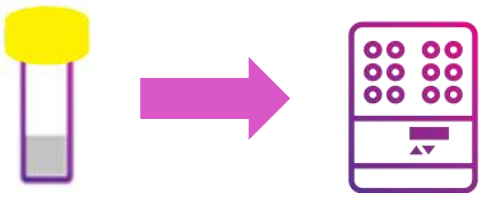


4. **Mix by vortexing for 10 secs and centrifuge sample briefly for 10 secs at 2,700-3,300 rcf [1]**



5. **Transfer 100µl of stool eluate to dilution buffer 2 vial (yellow cap)**

6. **Lyse sample at 105°C for 10min.**



[1] RCF = Relative Centrifugal Force. This is calculated based on the radius (r) of the rotor (in mm) and the centrifuge speed in revolutions per minute (rpm) as follows: $RCF = 1.12 \times r \times \left(\frac{rpm}{1000}\right)^2$

7. **Equilibrate the lysed sample at room temperature for 5min.**


8. **Centrifuge the sample for 10 secs @ 2700-3300 rcf.**


9. **Add 25µl of lysed sample to target and EC wells, vortex briefly to mix and centrifuge for 10 secs @2700-3300 rcf**

10. **Run on HG Swift**

## 8.1. Sample preparation

1. Unformed stool samples should be collected in suitable sterile container per applicable clinical guidelines and stored at 2-8°C for no longer than 4 days before testing.

2. For each stool sample to be tested, remove an individual dry swab from the kit. Swab the stool sample such that the swab is lightly coated and approximately ¾ of the swab surface is covered.

**Note 1:** *Use only the swabs supplied in the kit. Use of alternative swab types may impact test results.*

**Note 2:** *Overloading the swab with stool may impact the results of the test.*

3. Place the coated swab into a vial of Dilution Buffer 1 (green cap). Dip the swab up and down 10 times and then cut off the tip of the swab, taking care to wipe down the scissors after use. Close Dilution Buffer 1 vial and mix by vortexing for 10 secs. It is recommended to centrifuge the tube for 10 secs at 2,700-3,300 rcf to remove and droplets from the lid.

4. Using a calibrated micropipette, remove 100µl from Dilution Buffer 1 and add to a vial of Dilution Buffer 2 (yellow cap).

## 8.2. Lysis

**Note:** *Positive Controls do not require heat-treatment.*

1. Place each diluted sample in Dilution Buffer 2 in a dry bath incubator at 105°C for 10 minutes.
2. Remove and leave to equilibrate the sample to room temperature for 5 minutes.
3. Following the equilibration step, centrifuge the sample tube at 2,700-3,300 rcf for 10 seconds to remove condensate from the lid and top of the tube.

4. Add the lysed diluted sample to the reaction mix as soon as possible after the centrifugation step.

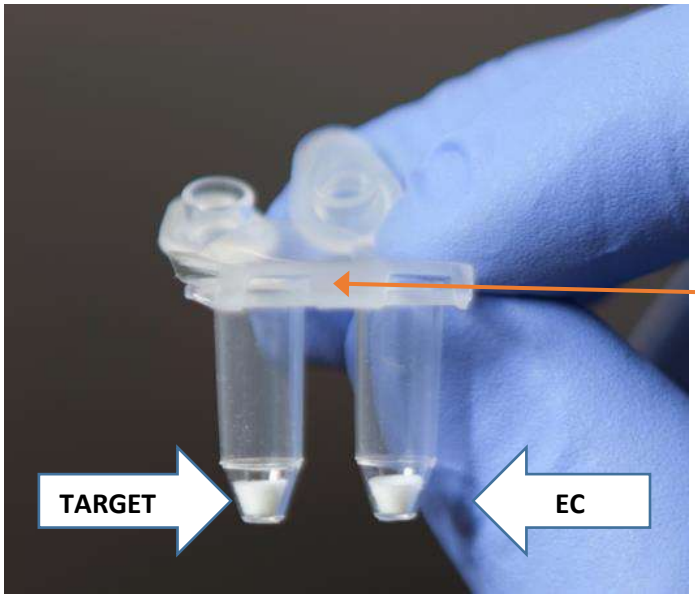
***Take every care to avoid cross-contamination when opening tubes after 105°C incubation, due to the possible presence of aerosol. It is recommended to change gloves after handling.***

## 8.3. Assay set up

1. Open the pouch containing the HG *C. difficile* reaction strips, tearing across from the notches on the pouch.

2. Remove the reaction strips, one strip per test to be conducted.

3. **It is vital to orient the reaction tubes in the correct direction**. When lids are facing away from the user, the **left-hand tube** in the strip contains the *C. Difficile* Target reaction mix and the **right-hand** tube contains EC reaction mix, as shown below:




4. If lyophilized pellets are sticking to the sealing film, tap the strip lightly until they fall to the bottom of the tubes and place the strip(s) in the HG Swift Set-up Rack in the correct orientation.

5. Peel off the plastic seal on the tubes carefully, taking care not to disrupt the lyophilized pellet and add 25µl of lysed sample to both the target (T) and endogenous control (EC) tubes.

6. Close the lid on each tube tightly by pressing firmly on the lids.

***It is critically important to ensure that lids are fully closed before commencing the run. Press lids down very firmly until a click or closure is evident.***

7. Reconstitute the reaction mixes by briefly vortexing briefly – minimise foaming of the solution. The vortexed reaction strips should then be briefly centrifuged (e.g. 10 sec at 2700-3300 rcf) to ensure that all liquid is returned to the bottom of the tubes, as shown below:

***Visually examine the reaction mix pellet after mixing to ensure lyophilized reaction mixture pellets are fully dissolved.***

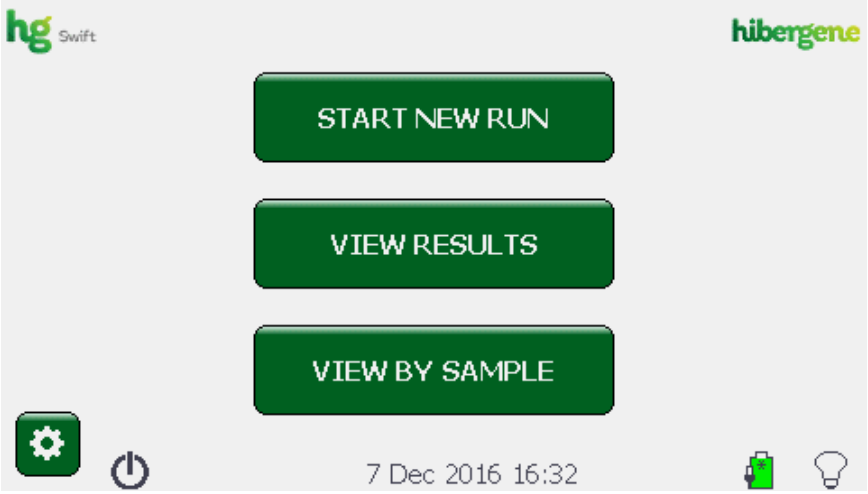
***Final reaction mixtures must be loaded and run on the HG Swift as soon as possible after reconstitution. Do not allow to stand for any longer than 10 minutes before starting the run.***

***After loading the strips on the instrument, press lids down again very firmly to ensure they are closed.***

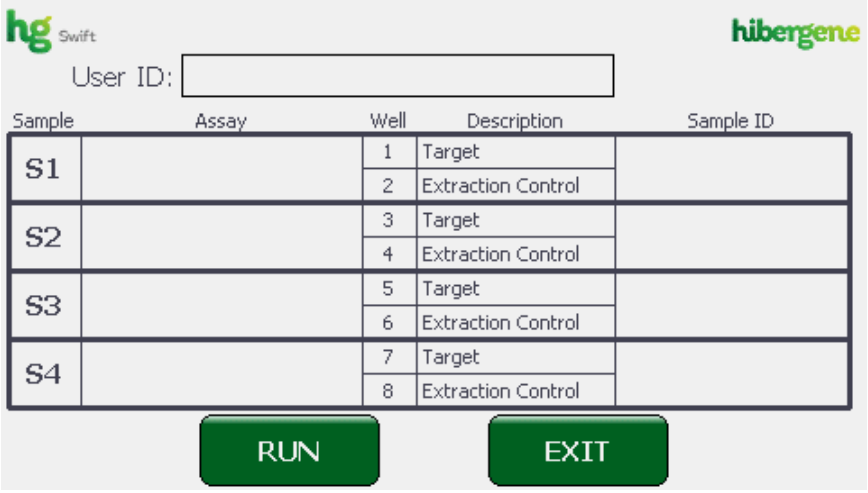
## 8.4. Run set up

1. Turn on the HG Swift using the power switch located at the back of the instrument.

2. Select START NEW RUN:




The Run Table will then be displayed:

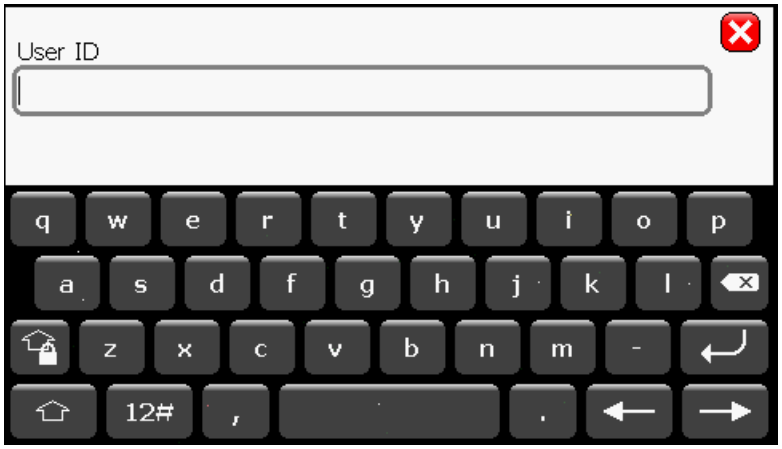


3. Load the reaction strip(s) onto the block of the HG Swift using a Strip Carrier, as shown below:

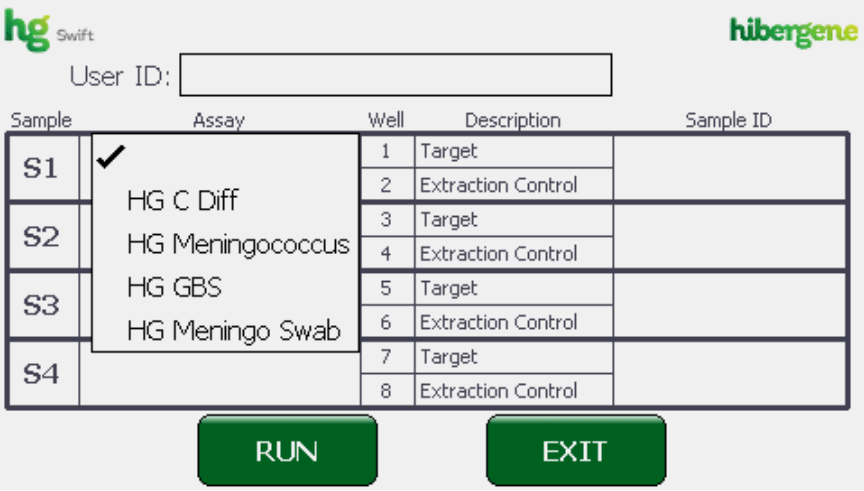
***The reaction strips must be loaded IN THE CORRECT ORIENTATION as shown above, with hinges towards the rear of the instrument.***

***Once reaction strips are loaded into the instrument, press down firmly on the lids a final time to ensure they are closed***

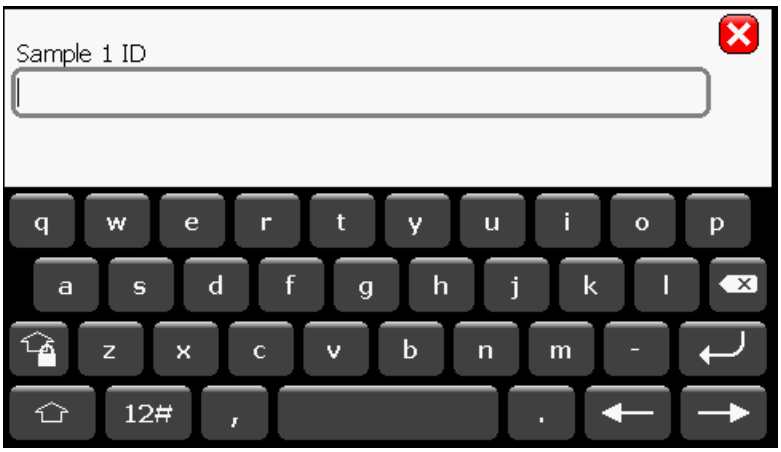
4. Enter the User ID using either the on-screen keyboard or a barcode reader attached to the HG Swift USB port:



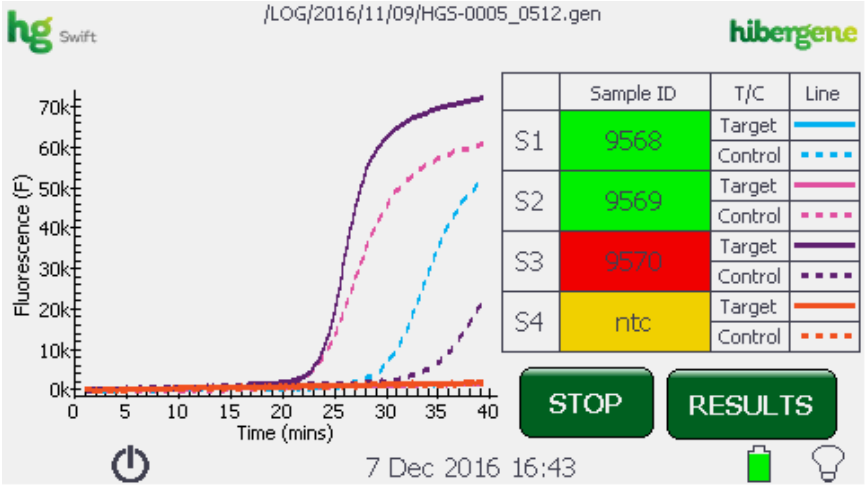
5. For each sample to be tested, select HG C Diff from the dropdown assay menu:



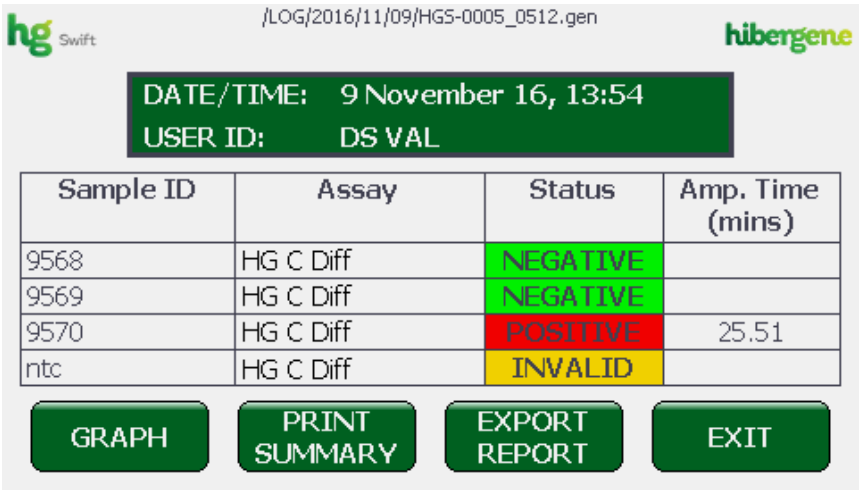

6. For each sample to be tested, enter the sample ID using either the on-screen keyboard or a barcode scanner:




7. Press RUN. The HG *C. difficile* run will take 40 minutes to complete. During the run the fluorescence of all samples will be displayed (see example below). The run may be aborted by pressing the STOP button:




8. During the run, any samples which generate positive amplification signals will be flagged as positive by a red fill colour in the Sample ID column. At any point during the run, the results screen may be accessed by pressing the RESULTS button:




When a run is in progress, samples which have already generated a positive result will be flagged as POSITIVE in the results screen, with other results listed as IN PROGRESS. The results screen may be printed - or a report generated - at any time during the run, using the PRINT SUMMARY / EXPORT REPORT buttons, to allow positive results to be reported out in real time.

# 9. Assay Results

The sample status which is displayed can be interpreted as follows:

| Displayed | Description |
|---|---|
| **POSITIVE** | *C. difficile* detected in the sample. For positive samples, the amplification time is displayed. This provides semi-quantitative information: earlier Amp Times reflect higher levels of target sequence. |
| **NEGATIVE** | *C. difficile* was not detected in the sample. |
| **INVALID** | Neither Target nor Extraction Control tubes amplified. This indicates possible issues with sample treatment, or the presence of inhibitors. See section 9.1 for instructions on retesting. |

## 9.1. Retesting Of Invalid Samples

Invalid results may be due to the presence of inhibitors in the sample. If desired, the following protocol may be used to further dilute samples for retesting:

- Add 30µl of the original sample eluate from Diluent Buffer 1 (green lid) into a new vial of Diluent Buffer 2 (yellow lid).
- Incubate vial at 105°C for 10 mins and retest per section 8..

**Note:** *Positive results obtained for initially invalid samples after dilution can be reported with confidence. However, negative results may be obtained if low levels of target in the sample are diluted below the assay LOD, and these results should be treated with caution.*

# 10. Result Printing & Storage

When a label printer is attached via the HG Swift USB port, a printed summary including User ID, Date and status of all samples can be obtained by pressing the PRINT SUMMARY button.

Pressing the EXPORT REPORT button generates a more detailed report which includes the amplification graphs. The report can be retrieved from the Reports folder on the HG Swift when a PC is attached (see HG Swift Instrument manual).

If PC is run as a sample, amplification times per the HGCDIFFC IFU will indicate acceptable performance.

If required, a No Target Control (NTC) may be included in the run by adding nuclease free water to both tubes of a strip – this should return an INVALID result and no amplification should be visible in either tube during the run. Alternatively, Dilution Buffer 1 or 2 may be added and run though the assay – this should return a valid negative result.

# 11. Performance Characteristics

## 11.1. Analytical Sensitivity

The Limit of Detection (LOD) of the assay - defined as the concentration of target for which the probability of detection is 95% - was determined using serial dilutions of plasmid DNA in buffer. 30 replicates at a range of concentrations were tested across 3 batches of HG *C. difficile* product. The LOD as determined by Probit analysis was as follows:

| Target | Limit of Detection (95% CI) |
|---|---|
| Plasmid DNA | 1.1 cps/µl (0.97 – 1.4 cps/µl) |

***Note: cps/µl = gene copies per microlitre***

To demonstrate the ability of the assay to detect multiple variants of *C. difficile*, negative stool was spiked with low doses of genomic extracts representing the *C. difficile* ribotypes and strains listed below. 8 replicates of each spiked sample were tested after treatment for 10 mins at 105°C. All replicates were detected.

| Ribotypes | Strains |
|---|---|
| 078 | 630 |
| 027 | 4206 |
| 017 | 4211 |
| 002 | 90556_M65 |

## 11.2. Analytical Specificity

A panel of 46 potentially cross-reactive organisms, including common human pathogens and bacteria closely related to *C. difficile* were tested across 3 batches of HG *C. difficile* product and no non-specific amplification was observed. The list of organisms tested was as follows:

| | | |
|---|---|---|
| *Bacillus cereus* | *Klebsiella pneumoniae* | *Pseudomonas aeroginosa* |
| *Enterococcus faecalis* | *Enterococcus faecalis* | *Enterobacter cloacae* |
| *Enterococcus faecium* | MRSA | *Aeromonas hydrophilia* |
| *Gardnerella vaginalis* | *Staphylococcus aureus* | *Clostridium sordelli* |
| *Lactobacillus crispatus* | *Micrococcus luteus* | *Yersinia enteroclitica* |
| *Streptococcus anginosus* | *Candida albicans* | *Proteus vulgaris* |
| *Streptococcus dysgalactiae* | *Streptococcus parasanquis* | *Salmonella enterica serovar enteriditis* |
| *Streptococcus equisimilis* | *Streptococcus intermedius* | *Salmonella enterica serovar Typhimurium* |
| *Streptococcus mitis* | *Escherichia coli* | *Clostridium perfringens* |
| *Streptococcus mutans* | *Citrobacter freundii* | *Clostridium septicum* |
| *Streptococcus pneumoniae* | *Serratia marcescens* | *Bacteroides fragilis* |
| *Streptococcus pyogenes* | *Sphingobacterium spiritivorum* | *Serratia liquefaciens* |
| *Streptococcus salivarius* | *Staphylococcus sciuri* | *Listeria monocytogenes* |
| *Streptococcus vestibularis* | *Acinetobacter lwoffii* | *Cambylobacter jejuni* |
| *Neisseria meningitidis* | *Staphylococcus epidermidis* | *Rotavirus* |
| *Faceal Adenovirus* | | |

In addition, BLAST analysis of the assay primer sequences did not identify any significant homology with the sequence of any databased organism which is likely to lead to non-specific amplification.

## 11.3. Relative Sensitivity and Specificity

To demonstrate the specificity of the assay relative to PCR, a panel of 153 unformed stool samples, collected and treated per section 0, were tested with both HG *C. difficile* and a commercial PCR method targeting the *tcdB* gene of *C. difficile*. All samples were tested with PCR and on each of 3 batches of HG *C. difficile* product.

6/153 samples returned invalid results upon initial testing. These were diluted and retested per section 9.1, and 4/6 samples gave valid results after dilution. After retesting to resolve any discrepant results, 1 sample which initially tested negative on PCR and positive with HG *C. difficile* was found to be positive by culture and excluded from the analysis. The final valid data set was as follows:

| | | **PCR** | |
|---|---|---|---|
| | | **Positive** | **Negative** |
| **HG *C. difficile*** | **Positive** | 21 | 4 |
| | **Negative** | 1 | 124 |

Based on this study, the relative sensitivity of HG *C. difficile* compared to PCR is 95.5% (21/22) and the relative specificity is 96.9% (124/128).

Positive samples were subjected to ribotyping analysis, and found to represent the following range of ribotypes: 001, 005, 014, 018, 023, 026, 078, 106 & 193.

### 11.4. Interferents

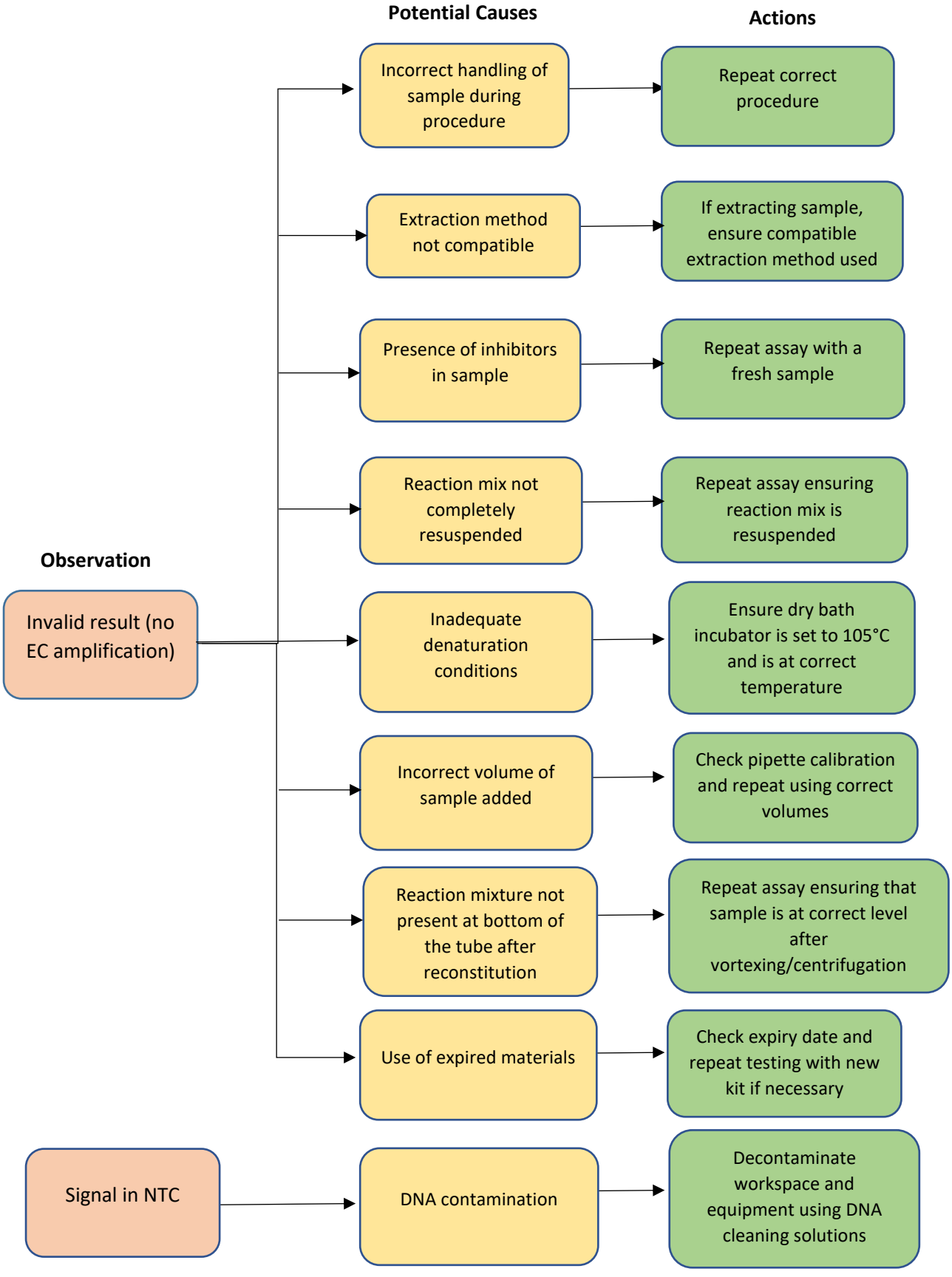
The impact of a range of potential inhibitors was assessed by loading positive and negative stool eluates with the substances listed below, and testing 4 replicates of each prepared sample. No significant levels of inhibition were detected:

| Substance | Detection Rate |
|---|---|
| Haemoglobin (3.2 mg/ml) | 100% (4/4) |
| Barium Sulphate (2.65 mg/ml) | 100% (4/4) |
| Metronidazole (12.5 mg/ml) | 100% (4/4) |
| Immodium (0.5 mg/ml) | 100% (4/4) |
| Gaviscon (0.5 mg/ml) | 100% (4/4) |
| Zantac (0.5 mg/ml) | 100% (4/4) |
| Calcium Carbonate (0.5 mg/ml) | 100% (4/4) |
| Mucin (3.3 mg/ml) | 100% (4/4) |
| Losec (0.5 mg/ml) | 100% (4/4) |
| Whole Blood (2.5% v/v) | 100% (4/4) |

## 12. Limitations of Use

- Any diagnosis of *C. difficile* infection must be made in conjunction with the entire clinical profile of the patient, including results of any other clinical laboratory tests which are available.
- The assay may not perform correctly if these instructions are deviated from, or if inhibitory substances are present in clinical samples.

# 13. Troubleshooting



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# 15. Interpretation of Symbols

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|---|---|
| IVD | In Vitro Diagnostic Medical Device |
| REF | Catalogue number |
| LOT | Batch number |
|  | Use-by date |
|  | Temperature limitation |
|  | Do not reuse |
|  | Manufacturer |
|  | Contains sufficient for <n> tests |
|  | Consult instructions for use (at www.hibergene.com) |
|  | IFU can be requested by phone if not accessible online |

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