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We use this protocol and it's working

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TaqMan Array Card (TAC) for enteropathogen detection

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ABSTRACT

Enteric infections are caused by a wide variety of pathogens and simultaneous infection with multiple pathogens can be common, thus making comprehensive diagnostic testing challenging. The TaqMan Array Card (TAC) is a 384-well real-time PCR format for TAC-compatible instrument platforms. A custom enteric TAC capable of detecting multiple targets on a single card for 8 samples has been developed, including viruses, bacteria, fungi, protozoa, and helminths. MS2 and phocine herpes virus (PhHV) are added to the fecal samples during nucleic acid extraction as external controls to monitor extraction and amplification. This procedure describes the a modified extraction protocol to isolate both DNA and RNA from fecal specimens using the QIAmp Fast DNA Stool Mini Kit, and the TAC setup protocol to detect enteropathogens.

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SAFETY WARNINGS

1. Always employ universal precautions.
2. Utilize techniques and /or equipment that will protect the operator from aerosols.
3. Do not put anything in the mouth during stool manipulations.
4. Wash hands after working with the stool sample and cultures.
5. Disinfect work surfaces before and after use.
6. As much as is feasible use disposable equipment, decontaminate reusable instruments after use.
7. Decontaminate biological waste before disposal.
8. Dispose of toxic chemical waste as prescribed by the institution.

Total nucleic acid extraction from stool samples.

1 **Sample preparation.** For fecal samples, weigh 180–220 mg stool or 200ul if liquid, into a 2 ml screw top tube that is compatible with the bead beater. For rectal swab sample, it is recommended to use low stopper, plastic applicator. After the shaft of the swab is snapped off, the bottom flocked portion is stored
frozen in a 2 ml screw top tube that is compatible with the bead beater. One blank negative control is included per batch of extraction.

2 **Sample lysis.**

2.1 **Prepare external control working solution.** Quickly spin the lyophilized external control vial (MS2 and PhHV mix) to bring the pellet to the bottom. Add 50 µl of standard diluent to each vial, mix well. Use 1 µl per sample extraction. Store the leftover at -80°C. **Don't use the reconstituted mix that has been frozen-thawed more than two times.**

2.2 **Prepare lysis buffer**. Mix InhibitEX Buffer thoroughly by shaking before use. If a precipitate has formed, Incubate at 37-70°C for ~15 minutes or until all precipitate has fully dissolved. Prepare fresh each day: Add external control working solutions to InhibitEX buffer; prepare only enough for N+1 samples to be processed that day: 1ml InhibitEX buffer +1ul external

control working solution is needed per sample
(e.g. For 10 samples 11ml InhibitEX+ 11ul external control working solution), mix well.

2.3 **Add beads. I**f the glass beads (acid-washed 212-300 µm, 50-70 U.S. sieve, Sigma G1277) have not been pre-aliquoted to empty tubes prior to aliquoting the samples: Add ~370 mg (one eppendorf tube capful) of beads to the aliquot of stool, or the flocked swab tip (or blank tube).

2.4 **Add lysis buffer.** Add 1 ml of the InhibitEX/external control solution into each specimen tube (or blank). Vortex for 1 min.

2.5 **Bead beating**. Bead beat at maximum speed for 2-3 minutes (instrument dependent, see below). Incubate
the suspension for 5 min at 95°C. Vortex for 15 s then centrifuge sample at full speed (approximately 20,000g) for 1 min to pellet the stool particles.
Note: Bead disruption for too long will overheat the sample and cause nucleic acid degradation, too little time is ineffective and will not enhance the extraction of DNA. Care should be taken in determining bead beating time for the instrument used. If immediately after bead beating the tube is hot, decrease the time. E.g., for Fastprep FP120 from Bio101 Thermo Electronic Corporation (Fisher NC9646109) - Bead disruption at maximum speed for 3 minutes is sufficient without over heating the preparation; For BioSpec Mini-Beadbeater (BioSpec 693, Mini-Beadbeater-8) - Bead disruption at maximum speed for 2 minutes is sufficient.

2.6 **Protease treatment**. Pipet 25 µl of proteinase K into a new 2 ml microcentrifuge tube (snap caps acceptable). Pipet 600 µl supernatant from step 2.5 into the 2 ml microcentrifuge tube containing proteinase K. Note: Do not transfer any solid material, if necessary centrifuge sample again.
Add 600 µl Buffer AL. Note: Do not add proteinase K directly to Buffer AL.
Vortex for 15 s, Mix thoroughly to form a homogeneous solution.
Incubate at 70°C for 10 min. Centrifuge briefly to remove dropsfrom the inside of the tube lid.

3 **Purification through the spin column.** Add 600 µl of ethanol (96–100%) to the lysate, and mix by vortexing. Centrifuge briefly to remove drops from the inside of the tube lid. Label QIAamp spin column lid and place in a 2 ml collection tube. Carefully apply 600 µl of the lysate to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Retain QIAamp spin column, place the column in a new 2 ml collection tube, and discard the collection tube containing the filtrate. Repeat two more times until all of the lysate (~1800 ul in total) has been through the spin column. Place the spin column in a new collection tube.

4 **Wash the spin column.** Open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate. Open the QIAamp spin column and

add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Retain QIAamp spin column, discard the collection tube
containing the filtrate. To eliminate the chance of possible Buffer AW2 carryover place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with filtrate. Centrifuge at full speed for 3 min. Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications.
Note: Buffer AW1 is supplied as a concentrate. Before using for the first time, add 25 ml ethanol (96–100%) as indicated on the bottle. Thoroughly mix before use. Buffer AW2 is supplied as a concentrate. Before using for the first time, add 30 ml ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle. Thoroughly mix before use.

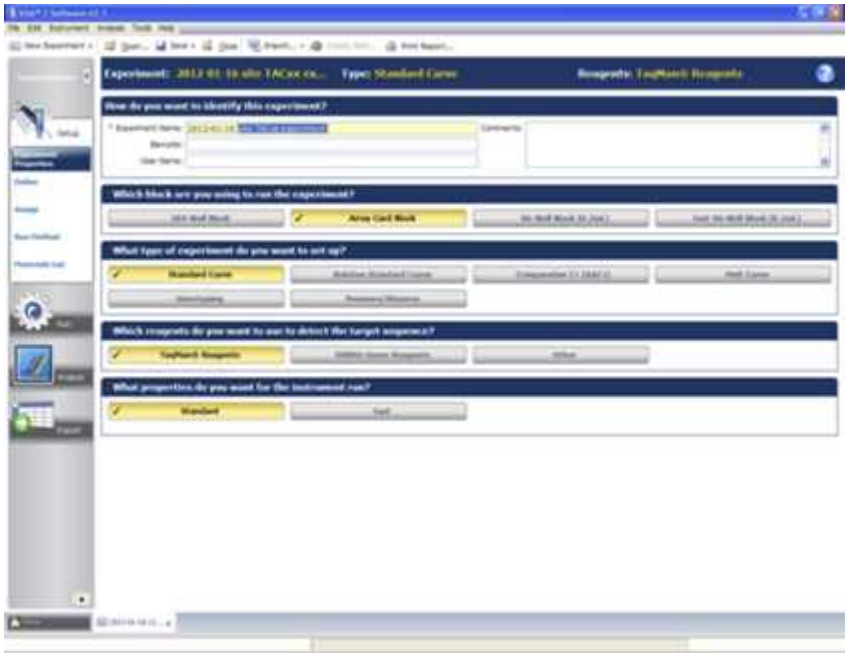
5 **Elute the total nucleic acid.** Transfer the QIAamp spin column into a labeled microcentrifuge tube. Open the QIAamp spin column and pipet 200 µl Buffer ATE directly onto the QIAamp membrane. Close the cap and incubate for 1-3 min at room temperature, then centrifuge at full speed for 1min to elute the TNA. Prepare
two tubes for aliquot storage. Pipet 100 ul of the extracted TNA into each tube.

6 **Storage of total nucleic acid.** Store at -70 to –80°C until testing.

TaqMan Array Card setup.

7 **Equipment preparation.** Ensure that the correct TaqMan Array Card block, heated cover, and loading tray are installed. Turn on the instrument and then the computer. Open the ViiA 7 or QuantStudio software programs as appropriate. Ensure that the computer is communicating properly with the instrument Click on the Instrument Console. When the computer properly connects with the instrument, the instrument icon will have a green check.

7.1 **Set up a new experiment "*From Template*"** using the lot specific template with targets, run method and analysis thresholds embedded. Confirm experiment Properties: Array Card Block, Standard Curve, TaqMan Reagents, Standard.



7.2 **Name the experiment** (the software automatically generates the name by date and time).

7.3 Click “Define” on the left panel, and **name the samples**. Note that the targets, fluorophores, passive reference dye have already been entered and assigned.

7.4 **Confirm Cycling Conditions** in the Run Method Screen:

Reaction volume: 1µL

Step	Temperature	Time
RT (1 cycle)	45°C	20min
Denaturation (1 cycle)	95°C	10min
PCR (40 cycles)	95°C	15sec
	60°C	1min (data collection)

7.5 **Save the experiment.** The file name is automatically generated based on the experiment name.

8 **Prepare qPCR reaction.** Prepare master mix for 8 samples:

Ag-Path-ID 2XRT-PCR buffer 425µL
Ag-Path-ID Enzyme mix 34µL
Aliquot 54µL of master mix into each of eight (8) 1.5mL microcentrifuge tubes. To each tube, add 20µL of total nucleic acid extract from **stool** specimens then supplemented with 26µL nuclease-free water, or
46µL of total nucleic acid extract from **rectal swabs**, or extraction blanks, or nuclease-free water (for NTC).
Mix gently, then centrifuge the tubes to eliminate air bubbles from the mixtures.

9 **Load the TaqMan Array Card.** Allow the TAC cards to reach room temperature in the original packaging. Place the card on a lab bench, with the foil side down. Transfer 100µL of each PCR reaction mix into the fill port. Hold the pipette in an angled position into the fill port (the larger hole on the left arm) and slowly dispensing the mix so that it sweeps in and around the fill reservoir toward the vent port without introducing air bubbles. Be careful when pushing the pipette plunger to its end as to avoid pushing the reaction mix out of the fill reservoir via the vent port or introducing bubbles.

10 **Centrifuge the TaqMan Array Card.**

10.1 Set the bucket type: Previous-Generation centrifuges: 15679; Current-Generation Centrifuges: 3618.
Confirm the proper centrifuge settings.

Parameter	EASYSet (touchpad)	QUIKSet (knob-operated)
Up Ramp rate	9	3
Down Ramp rate	9	N/A
Rotational speed	1,200 rpm (331 xg)	1,200 rpm
Centrifuge time	2 X 1 min	2 X 1 min

10.2 Insert TaqMan Array Card into the array holder, ensuring that the filled reservoirs should project upwards out of the array holder; The reaction wells face the same direction as the “This Side Out” label on the array holder. Use blank arrays (*i.e.* empty cards with no spotted primers/probes) or used arrays to fill any remaining positions in the array holder. Failing to do so will impair the sample loading. Place a filled array holder in the bucket so that the “This Side Out” label faces the front of the bucket.

10.3 Place a loaded bucket onto an open rotor arm of the centrifuge. Make sure the bucket can swing easily within its slotted position on the rotor arm. The manufacturer recommends

running the centrifuge with all
four buckets, even if only two buckets contain cards. Make sure the buckets and their contents are balanced.

10.4 Centrifuge 1 minute. Wait for the centrifuge to stop, then repeat so that the cards are centrifuged for a total
of two consecutive, 1-min spins.

10.5 Remove the buckets from the centrifuge, then remove the array holders from the buckets. Remove the TaqMan array card(s) from the holders. Examine the TaqMan Array card(s) to determine whether filling is complete. A small amount of sample will remain in the fill port, but it should be a uniform volume from reservoir to reservoir. If there is excess reaction mix in a fill reservoir, centrifuge the card again for 1 additional minute. If the reservoir filling is still not complete, do not centrifuge again. Proceed with the card run but void the results for the affected sample. Excessive centrifugation speeds and times may deform the card, so do NOT exceed 1200rpm or accumulated centrifugation times of more than 3 minutes. If a fill reservoir is completely drained, it is possible that some wells are not filled properly. Proceed with the card run but void the results for the affected sample.

11 **Seal the TaqMan Array Card.** The sealer (“staker”) isolates the wells of a card after loading with reaction mix. The sealer uses a precision stylus assembly (“carriage”) to seal the main fluid distribution channels of the array. Proper operation of the sealer is critical to the successful use of the TaqMan Array Card. Place
the sealer on a sturdy lab bench, approximately waist high so that it can be easily used. Make sure the carriage sits in the “starting position” which is closest to you. Never insert a card into the sealer if the carriage is NOT in its starting position. The card will be irreparably damaged if the carriage is moved across
it in the wrong direction.

11.1 Insert a TaqMan Array Card into the sealer. Orient the card in the proper direction over the sealer’s insert plate, **foil side up.** The fill ports should be at the farthest end of the sealer base (away from you). Line
up the card’s rear pin groves to the stylus pins on the sealer. Gently place the card on top of the insert plate and ensure that the front end of the card is held securely in place by the spring clips. Gently push the card until it is seated securely in the insert plate. When properly seated, the card’s foil surface should be level with the base of the sealer. The four spring clips ensure that the card is held in the proper position.

11.2 Push the carriage across the base of the sealer in the direction of the arrow at the far end labeled with “Push to stake”. Use a **slow, steady** and deliberate motion to push the carriage across the entire length of the card until the carriage reaches the mechanical stops. It is important to avoid moving the carriage rapidly across the card.

11.3 Remove the sealed card by grasping its sides and lifting it off the sealer’s insert plate. In the

middle of the sealer's insert plate, there is thumb slot to help you easily access one side of the card. Inspect the card for proper sealing. The indentations from the stylus assembly should match up with the card's main channels. If the indentations do not match up or if the foil is in any way damaged, do not use the card.

11.4 Return the carriage to its starting position on the base of the sealer.

12 **Trim off the fill reservoirs.** Using scissors, trim the fill reservoirs from the card. Use the edge of the card's
carrier as a guide.

13 **Run the experiment.** Place the sealed card in the instrument with well A1 at the top left corner of the
tray and the notched corner at the top right, with the bar code facing toward the front of the instrument.
Within the software, click "Run" on the left panel, then the arrow next to "START RUN", select the instrument. You should be prompted to select a location to save the run file if the experiment hasn't
been saved.