VANSCAN™ Typhoid - RT PCR Test

1. Intended Use

VANSCAN **Typhoid RT PCR Test** is a molecular diagnostic test for the qualitative detection of Salmonella typhi and Salmonella paratyphi in human blood.

2. Introduction

Typhoid and paratyphoid fevers are commonly grouped together under the collective term - 'enteric fever'. Typhoid fever is a serious illness caused by Salmonella typhi, a gram-negative bacterium while paratyphoid fever is caused by Salmonella paratyphi A, B, or C subtypes.

Typhoid fever is prevalent in developing countries. In the year 2019, 9.2 million cases of Typhoid fever and 110,000 deaths were reported worldwide, with the highest incidence in the South East Asian, Eastern Mediterranean and African regions. On an average, 12.5 million people are infected with the illness every year. Typhoid and paratyphoid infections are transmitted mainly by the fecal-oral route. The infection is contracted by eating or drinking contaminated food or water or by coming in contact with patients of typhoid fever. The bacteria are carried in the bloodstream and the intestinal tract of infected persons.

Early and accurate diagnosis of typhoid fever is imperative to initiate treatment by administering the right antibiotics and reducing the risk of Antimicrobial Resistance (AMR). Current methods of diagnosis include serology tests like the conventional WIDAL test and Rapid Card Tests and the blood culture test. The WIDAL test detects antibodies to Salmonella typhi and paratyphi in human serum and is positive only from the second week of onset of symptoms. The WIDAL test is more than 100 years old and the results obtained with it are largely unstandardized and inconclusive. Rapid Card Tests detect the presence of IgM and /or IgG antibodies to S.typhi. Both these tests have been widely reported to suffer from the limitations of poor sensitivity, specificity and reliability. The current gold standard is the blood culture test, however, it is cumbersome and time consuming. The results of a blood culture test can be affected by intake of antibiotics.

VANSCAN **Typhoid RT PCR Test** is a nucleic acid (bacterial DNA) based diagnostic assay for the detection of Typhoid using a patented technology (Patent No. 355208). It uses S. typhi and S. paratyphi specific signature sequence as biomarker along with novel primers and probes. The VANSCAN **Typhoid RT PCR Test** uses DNA isolated from the whole blood of infected humans and has been found to be highly sensitive and specific.

3. Principle of the Test

VANSCAN **Typhoid RT PCR Test** works on the principle of nucleic acid amplification through Real Time Polymerase Chain Reaction (RT PCR), with the help of a specific set of primers targeting a signature DNA and its detection by a specific probe. The probes contain a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence Resonance Energy Transfer (FRET). The probe hybridizes with one of the chains of the amplified fragment, and is degraded during the extension cycle. As a consequence, the fluorophore is separated from the quencher resulting in the emission of FRET. The total fluorescence of the reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. The fluorescent signal is measured in each cycle of the reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle value is inversely proportional to the number of DNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples. In VANSCAN **Typhoid RT PCR Test**, the target sequence has been taken from the 'omp' gene encoding the 'outer membrane protein'. The sequence is specific and highly conserved for Salmonella typhi and Salmonella paratyphi (Patent No- 355208).

VANSCAN **Typhoid RT PCR Test** is optimized with an internal control of human gene, RNAse P.

4. Kit Components for 24 Test Pack Size

a) Enrichment Buffer (provided in pre-filled tubes in separate box)	-	4 ml
b) Enzyme Master Mix	-	250 µl
c) Primer Probe Mix	-	50 µl
d) Positive Control (PC)	-	20 µl
e) Nuclease Free Water (NFW)	-	100 µl
f) RT PCR Tubes & Caps	-	3 No.
g) Instructions For Use (IFU)	-	1 No.

5. Storage & Stability

- Store the kit and its contents at - **20°C** up to the expiry date printed on the labels.
- Allow reagents to be thawed completely on ice or at 4°C prior to use.
- Return all the kit components immediately to - **20°C** after use.
- Repeated thawing and freezing of kit reagents (> 4 times) should be avoided as it reduces assay sensitivity.
- Kit reagents are stable till the end of the expiry date indicated on the box when stored at - **20°C**.

6. Materials Required but Not Provided

a. Lab Instruments: Any Real Time PCR instrument calibrated for FAM, HEX and BHQ1 dye.

CFX 96 BioRad RT PCR machine and Thermo Fisher RT PCR machines can be used with this kit.

b. Basic Lab Materials: Biosafety cabinet Type-2, vortex mixer, cooling centrifuge (block 96 x 0.2 mL tubes) and refrigerator (-20°C to -10°C), calibrated variable micropipettes-precise single and multichannel adjustable pipettors (1.00 µL to 1,000.0 µL), PCR vials or 96 well format block.

c. 1.5 ml tube, 0.2 ml and 1.5 ml microcentrifuge tube stand, cooling block.

d. Sterile nitrile gloves, facemask, head cap, lab coats etc.

e. Blood DNA Extraction Kit.

7. Specimen Collection and Storage

2-3ml of whole blood samples should be collected from the patients preferably in K_3EDTA tubes. These tubes having whole blood specimens should be placed immediately on refrigerant gel-packs or in the refrigerator for no longer than 48 hours. Alternatively, specimens be frozen at -80°C.

8. DNA Extraction

DNA can be extracted from the collected blood sample either using an automated nucleic acid extractor or by manual methods (Spin Column based Kits) as per the instructions and protocol provided by the DNA extraction kit manufacturer. Following extraction kits are recommended to be used:

- Qiagen 51104, 56304, 69504.
- Any standard blood DNA isolation kit.
- Quality of extracted DNA from whole blood is the crucial material for the VANSCAN **Typhoid RT PCR Test** performance. Thus, extracted DNA must be used immediately or stored at -80°C till further use.

9. Warnings and Precautions

- VANSCAN **Typhoid RT PCR Test** should be used for *in- vitro* diagnostics use only.
- Read the IFU provided carefully before starting the assay.
- Laboratory personnel should wear appropriate personal protective gears and should be trained on the RT PCR machine.
- Specimens should always be treated as infectious and/or biohazardous. They should be handled in accordance with safe laboratory procedures and in a BSL-2 biosafety hood while adhering to BSL-2 practices.
- Avoid microbial and nuclease (DNAse/RNAse) contamination of the components of the kit.
- Always use DNAse/ RNAse-free disposable pipette tips with aerosol barriers.
- Use separated and designated working areas for (i) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities.
- Workflow in the laboratory should proceed in a unidirectional manner.
- Do not open the reaction tubes/plates post amplification in order to avoid contamination with amplicons.
- Do not use components of the kit that have passed their expiry date.
- Discard sample and assay waste according to the local safety regulations.
- NFW is prone to contamination. It should be handled with utmost precautions.

10. Test Procedure

a. Take out all the vials from the kit and thaw on ice.

b. Depending upon the required number of reactions, mix all the components carefully as described in Table 1 and aliquot in the respective PCR tubes provided.

c. Add 8 µl of extracted DNA (Instruction No.8) from unknown samples respectively in separate tubes of the PCR strips.

d. Add, 8 µl PC and 8 µl of NFW from the kit in respective tubes as Positive and No Template Controls (NTC).

e. Use white, RT PCR DNAse/RNAse free strips provided in the kit for accurate results.

f. After adding samples and controls in the tubes, cover them properly with the caps provided.

g. Give a short spin for pulling down any component stuck to the walls of the tubes and for the removal of any air bubble.

Table 1

Contents	PC Well	NTC Well	Sample Well
Enzyme Master Mix	10 µl	10 µl	10 µl
Primers/Probe Mix	2 µl	2 µl	2 µl
PC / NFW / Sample	8 µl	8 µl	8 µl

Table 2

Sr.No.	Components	Temperature	Time	Cycle
1.	Denaturation of DNA	95°C	03 min	1
2.	Denaturation	95°C	10 sec	40
3.	Annealing	58°C	15 sec	
4.	Extension & Plate Read	72°C	15 sec	

Note: The final Reaction Volume of each test should be 20 µl (12 µl Master Mix + 8 µl Sample).

h. Set the RT PCR machine in accordance with the protocoldescribed in Table 2.

I. Select the two-fluorescence channels (FAM and HEX) for each sample selection in the software of the RT PCR machine and set passive reference to none.

j. Any standard RT PCR machine which has the FAM (Green) and HEX (Yellow) channels can be used e.g. BioRad, Thermo Fisher (Quant Studio), Agilent

11. Quality Check & Interpretation of Results

Table 3

	Reaction	Ct Value of Target (FAM labelled)	Ct Value of Internal Control (HEX labelled)	Inference
Quality Check	Positive control	≤38	≤38	Performance of kit is acceptable
	No Template Control	NA* or ≥ 38	NA* or ≥ 38	
Interpretation of Results	Positive test sample	≤38	≤38	Positive Result
	Negative test sample	NA* or ≥38	≤38	Negative Result

*Machine may display Not Applicable (NA) when there is no signal detected.

In a few positive cases where the bacterial load in the sample is very low, the test could give a negative result. In such cases the test should be repeated with an additional step to enrich the bacteria in the sample, as follows:

12. Enrichment of Bacteria in the Sample

It is recommended that sample enrichment be carried out prior to the DNA extraction from the whole blood. This process will serve to enhance the number of infectious bacteria (if present) in the blood sample for DNA extraction.

Step -1: Take 2 ml of whole blood (EDTA) in a test tube and add 4 ml of Enrichment Buffer provided in the kit.

Step -2: Incubate at room temperature (20 - 25°C) for 10 minutes.

Step -3: Centrifuge the tube from Step -2 at 12,000 rpm for 5 minutes at room temperature (20 - 25°C).

Step -4: Discard the supernatant and resuspend the pellet in the Lysis Buffer of the DNA extraction kit.

Step -5: Extract the DNA as per the instruction provided by themanufacturer of the DNA extraction kit.

Step -6: Repeat the protocol as described under the Test Procedure (Instruction 10).

13. Procedural Limitations

1. Risk of false negative test results is possible in the events of antibiotic intake by the patient before the collection of the sample, inadequate numbers of bacteria in the blood stream for amplification and any procedural errors.
2. VANSCAN **Typhoid RT PCR Test** is a qualitative test and does not provide the quantitative test results.

13. Performance Characteristics

The VANSCAN **Typhoid RT PCR Test** kit was validated at the following sites: Table 4

Table 4

Validation Site	Culture vs VANSCAN Typhoid RT PCR Test		Culture vs VANSCAN Typhoid RT PCR Test	
	Culture Positive	RT PCR Positive	Culture Negative	RT PCR Negative
Regional Centre for Biotechnology (DBT-Lab), Faridabad	15	15	7	7
Graphic Era University, Dehradun	84	84	*153	148
	Reference Kit	RT PCR Positive	Reference Kit	RT PCR Negative
NABL Accredited Lab, Jaipur	8	8	14	14
Total	**107**	**107**	**174**	**169**

*Out of the 153 culture negative samples tested, 5 samples were found to be positive with RT PCR (also shown in Figure 1).
Thus, VANSCAN **Typhoid RT PCR Test** was found to be more sensitive than the culture method.

Culture-positive and Culture-negative typhoid samples were compared with VANSCAN **Typhoid RT PCR Test** as summarized below in Figure 1.

Figure 1

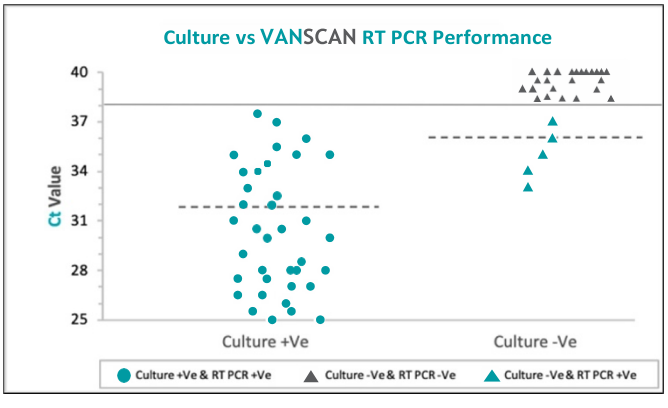

Figure 1: The solid line indicates the Ct cutoff value of ≤38 representing a positive result by RT PCR. The dashed lines indicate the median Ct value for each group.

The Sensitivity and Specificity of VANSCAN **Typhoid RT PCR Test** versus Blood Culture (Table 4), other reference kit (Table 4) and no-cross reactivity with other pathogens (Table 5) were analyzed and validated using a total of 332 samples which were distributed as follows (Table 5):

Table 5

True Positive by RT PCR	True Negative by RT PCR	No-Cross Reactivity by RT PCR (n=82)	
107 + 5 (Missed by Culture)	169	Healthy Subjects	22
		Bacterial Infection	15
		Mycobacterial infection	10
		Dengue Infection	15
		Chikungunya Infection	02
		Malaria (PV) Infection	10
		Malaria (Pf) Infection	08
112	**169**		**82**

Load of Detection

- VANSCAN **Typhoid RT PCR Test** detects infectious bacterial DNA of typhoid with **a detection limit of ≤ 10 pico-gram of Bacterial Genomic DNA.**
- With reference to the Colony Forming Unit (CFU) used as gold standard technique of blood culture, 1- 10 CFUs of bacteria in whole blood are detectable by the VANSCAN **Typhoid RT PCR Test.**

14. Bibliography

1. Hancuh M, Walldorf J, Minta AA, et al. 2023. Typhoid Fever Surveillance, Incidence Estimates, and Progress Toward Typhoid Conjugate Vaccine Introduction - Worldwide, 2018-2022. MMWR Morb Mortal Wkly Rep.72:171-176.
2. Bhan MK, Bahl R, Bhatnagar S. 2005. Typhoid and paratyphoid fever. Lancet. 366(2):749-762.
3. Buckle GC, Walker CLF, Black RE et al. 2012. Typhoid fever and paratyphoid fever: systematic review to estimate global morbidity and mortality for 2010. J Glob Health. 2:010401.
4. Bahl R, Sinha A, Poulos C, et al. 2004. Costs of illness due to typhoid fever in an Indian urban slum community: implications for vaccination policy. J Health Popul Nutr. 22: 304-10.
5. Crump JA, Luby SP, Mintz ED. 2004. The global burden of typhoid fever. Bulletin of the World Health Organization. 82:346-353.
6. GBD 2017 Typhoid and Paratyphoid Collaborators. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis. 2019 Apr;19(4):369-381.
7. Loz_ano R, Naghavi M, Foreman K, et al. 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 380: 2095- 128.
8. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, Bhutta ZA, Canh do G, Ali M, Shin S, et al. 2008. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bulletin of the World Health Organization. 86:260-268.
9. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. 2002. Typhoid fever. The New England Journal of Medicine. 347:1770-1782. doi: 10.1056/NEJMra020201.
10. Pilecky M, Schildberger A, Orth-Höller D, Weber V. Pathogen enrichment from human whole blood for the diagnosis of bloodstream infection: Prospects and limitations. Diagn Microbiol Infect Dis. 2019 May;94(1):7-14.
11. Trung NT, Hien TT, Huyen TT, Quyen DT, Van Son T, Hoan PQ, Phuong NT, Lien TT, Binh MT, Van Tong H, Meyer CG, Velavan TP, Song le H. Enrichment of bacterial DNA for the diagnosis of blood stream infections. BMC Infect Dis. 2016 May 31;16:235.
12. Loonen AJ, Bos MP, van Meerbergen B, Neerken S, Catsburg A, Dobbelaer I, Penterman R, Maertens G, van de Wiel P, Savelkoul P, van den Brule AJ. Comparison of pathogen DNA isolation methods from large volumes of whole blood to improve molecular diagnosis of bloodstream infections. PLoS One. 2013 Aug15;8(8):e72349.

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