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Unzipping Genes

HIMEDIA®

# MBT147 E. coli O157: H7 Water Detection Kit (Real time Probe Based PCR)

**Kit Contents for DNA Purification**

| Product Code | Reagents provided | MBT147 | |
|---|---|---|---|
| | | **50 Preps** | **100 Preps** |
| DS0202 | Water Lysis Solution (WL) | 60 mL | 120 mL |
| DS0066 | Inhibitor Removal Solution (IRSH) | 15 mL | 30 mL |
| DS0203 | Binding Solution (WBS) | 40 mL | 80 mL |
| DS0012 | Wash Solution Concentrate (WS) | 8 mL | 16 mL |
| DS0204 | Wash Solution (WT) | 40 mL | 80 mL |
| DS0040 | Elution Buffer (ET) [10mM Tris-Cl, pH 8.5] | 6 mL | 12 mL |
| DBCA020 | Hi-Water Bead Tube | 52 nos. | 104 nos. |
| DBCA03 | HiElute Miniprep Spin Column (Capped) [in DBCA016 Collection Tube] | 55 nos. | 110 nos. |
| DBCA016 | Collection Tube (Uncapped), Polypropylene (2.0 mL) | 55 nos. | 110 nos. |
| PW1139 | Collection Tube, Polypropylene (2.0 mL) | 150 nos. | 300 nos. |

**Kit Contents for Real time Probe Based PCR**

| Components | Product code | Reagents provided for (reactions)* | |
|---|---|---|---|
| | | **50R** | **100R** |
| Hi-Quanti 2X Realtime PCR Master Mix | MBT180 | 750 µL | 1.5 mL |
| E. coli O157:H7 Primer-Probe Mix | DS0739 | 70 µL | 140 µL |
| Internal Control Primer-Probe Mix | DS0741 | 70 µL | 140 µL |
| Internal Control E DNA | DS0619 | 100 µL | 200 µL |
| Molecular Biology Grade Water for PCR | ML065 | 4 mL | 8 mL |
| Positive Control (E. coli O157:H7 DNA) | DS0353 | 300 µL | 600 µL |

## Introduction

*Escherichia coli* O157: H7 is a rod-shaped, gram-negative, aerobic, non-fermentative bacterium that causes hemolytic colitis and hemolytic uremic syndrome. The main mode of infection is through food, untreated water and unpasteurized milk. Transmission is via the fecal-oral route, and most illness has been through distribution of contaminated leafy green vegetables. *E.coli* O157:H7 was identified as a pathogen in 1982 following its association with two food-related outbreaks of gastrointestinal illness. This organism is characterized by Endotoxin shock as one of the lethal manifestation of infection. *E.coli* O157:H7 is known by its somatic (cell wall) antigen (O157) and its flagella antigen (H7). *E.coli* O157:H7 is also known to produce Shiga-like toxins, also known as vero-toxins which cause severe symptoms like diarrhea, fatigue etc. Specific and faster methods for detection of foodborne pathogens, such as Real-Time PCR, are the need of an hour. These techniques help to detect targeted pathogens quickly; this early and precise detection helps to take further actions.



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The polymerase chain reaction (PCR) with real time application, alternatively termed as Real Time PCR or quantitative PCR (qPCR) is considered the most adaptable and prevalent nucleic acid based technique. It is a highly specific and sensitive alternative method to standardize culture isolation, especially when rapid results are required. Over the past few years, molecular techniques based on 16S rRNA gene besides other genetic markers have been developed to analyze bacterial communities in environmental samples.

**NOTE**: HiMedia's E. coli O157:H7 Water Detection Kit (Real time Probe Based PCR) is for *in-vitro* use only.

**Intended Use**
Recommended for sensitive and specific detection of *E. coli O157:H7* in water samples.

**Sample Source:** Water samples

**Specimen collection and Handling**
Follow appropriate techniques for handling specimens; after use, contaminated materials must be sterilized by autoclaving before discarding. Standard precautions as per established guidelines should be followed while handling clinical specimens and items contaminated with blood and other body fluids. Safety guidelines may be referred in individual safety data sheets.

**Materials needed but not provided**

**For DNA Purification**
- 0.22µmfilter paper
- Filtration system
- HiMedia Product code : SF86 [S.S. Sterility Test Manifold System], SF84 [S.S. Sterility test Filter Holder])
- Vacuum Source (for filtration of water sample)
- Sterile forceps
- Pipettes and sterile tips (barrier / non-barrier) of variable volumes
- Tabletop Microcentrifuge (with rotor for 2.0 mL tubes)
- Ethanol (96 – 100%) (For dilution of Wash Solution Concentrate)
- Ice bucket (for 4°C incubation)
- Molecular Biology Grade Water (HiMedia Product code: ML024)
- PCR tubes (Product code PW1255) or PCR Strips (Product code: PR17) or PCR Plates (Product code: PR2 / PR3 / PR19)
- Insta Q Real Time PCR System (Product Code: LA1012/LA1073/LA1023/LA1024/LA1074)

**For PCR assay:**
- PCR tubes (Product code PW1255) or PCR Strips (Product code: PR17) or PCR Plates (Product code: PR2 / PR3 / PR19)
- Insta Q Real Time PCR System (Product Code: LA1012/LA1073/LA1023/LA1024/LA1074)
- Barrier Micropipette Tips
- Micropipettes

**Storage and Shelf life**
Store the DNA Purification Kit between 15-25°C except certain components as specified on each labels. Store the PCR kit reagents at -20°C. Under recommended condition, the provided kit has a shelf-life of 12 months.

Repeated thawing and freezing of PCR reagents should be avoided, as this may reduce the sensitivity. If the reagents are to be used multiple times, we recommend storing reagents as aliquots to avoid repeated freeze and thaw. Degradation of sample DNA specimens can also reduce the sensitivity of the assay. HiMedia does not recommend using the kit after the expiry date stated on pack.

**Principle**

E. coli O157:H7 Water Detection Kit (Real time Probe Based PCR) provides a convenient and rapid method for detection of *E. coli O157:H7* from water samples. The kit allows rapid isolation and DNA purification procedure using the miniprep spin columns that comprises of three steps viz.,

- Adsorption of DNA to the membrane,
- Removal of residual contaminants and
- Elution of pure genomic DNA to yield high quality DNA.

For DNA purification, the water sample is passed through a 0.22 µm filter membrane and the micro-organisms present in the water are trapped. The membrane is then placed in a Hi-Water Bead tube for thorough lysis in presence of a Lysis Buffer. After the inhibitor removal step, the DNA is purified using HiElute Miniprep Spin Column (Capped) for further downstream analysis by qPCR. Following the purification, qPCR for rapid, sensitive and specific detection of *E. coli O157:H7* is designed that targets the **outer membrane protein (eae)** gene for *E. coli O157:H7* detection.

**HiElute Miniprep Spin Column (Capped) [DBCA03]**

HiElute Miniprep Spin Column (Capped) is based on the advanced silica binding principle presented in a microspin format. The present system efficiently couples the speed and reversible nucleic acid-binding properties of the advanced silica gel membrane with versatility of spin column technology to yield larger quantity of DNA with highest purity. The use of spin column facilitates the binding, washing and elution steps thus enabling multiple samples to be processed simultaneously. This column eliminates the need for alcohol precipitation, expensive resins, and harmful organic compounds such as phenol and chloroform, otherwise employed in traditional DNA isolation techniques. DNA binds specifically to the advanced silica-gel membrane while contaminants such as PCR inhibitors which include divalent cations and proteins are completely removed in two efficient wash steps, leaving pure genomic DNA to be eluted in the buffer provided with the kit.

**Elution**

The yield of genomic DNA depends on the sample type and the number of cells in the sample. A single elution with 100 µL of Elution Buffer (ET) will provide sufficient DNA to carry out multiple amplification reaction. Elution with volume less than 100 µL will increase the final DNA concentration, but will reduce the overall DNA yield.

**Concentration, yield and purity of DNA**

Spectrophotometric analysis and agarose gel electrophoresis will reveal the concentration and the purity of the genomic DNA respectively. Elution Buffer (ET) is used to dilute samples and to calibrate the spectrophotometer. The absorbance is measured at 260 nm, 280 nm, and 320 nm using a quartz microcuvette. Absorbance readings at 260 nm should range between 0.1 and 1.0. The 320 nm absorbance is used for correction of background absorbance. An absorbance of 1.0 at 260 nm corresponds to approximately 50 µg/mL of DNA. The $A_{260}$–$A_{320}$ /$A_{280}$–$A_{320}$ ratio should be 1.6 – 1.9. Purity is determined by calculating the ratio of $A_{260}$–$A_{280}$. DNA extracted using HiMedia's E. coli O157:H7 Water Detection Kit (Real time Probe Based PCR) is free of protein and other contaminants that can inhibit PCR or other enzymatic reactions.

Concentration of DNA sample (µg/mL) = 50 x $A_{260}$ x dilution factor.

**General Preparation Instructions**

1. **Thoroughly mix reagents**
   Examine the reagents for precipitation. If any kit reagent forms a precipitate (other than enzymes), warm at 55-65°C until the precipitate dissolves and allow cooling to room temperature (15-25°C) before use.

2. **Ensure that clean & dry tubes and tips are used for the procedure.**

3. **Dilute Wash Solution Concentrate (WS) (DS0012) as follows:**

| Number of Preps | Wash Solution Concentrate (WS) | Ethanol (96-100%) |
|---|---|---|
| 50 | 8 mL | 32 mL |
| 100 | 16 mL | 64 mL |

**Centrifugation**

All centrifugation steps are carried out in conventional laboratory centrifuge with fixed angle rotor. The tubes provided with the kit are compatible with almost all laboratory centrifuges and rotors. All centrifugation steps are performed at room temperature and are given in g, the correct rpm can be calculated using the formula:

$$RPM = \sqrt{RCF/1.118 \times 10^{-5}\, r}$$

where *RCF* = required gravitational acceleration (relative centrifugal force in units of g); *r* = radius of the rotor in cm; and *RPM* = the number of revolutions per minute required to achieve the necessary *g*-force.

**Procedure**

**I) <u>DNA Purification from Water Sample</u>**

1) Filter 1000 mL of water sample through a pre-sterilized 0.22 µm filter paper to trap the microorganisms.

2) Aseptically remove the filter paper using sterile forceps, gently roll the paper and place it inside the Hi-Water Bead tube (provided), such that the top side of the filter paper faces inward.

3) **Lysis**
   Add **1 mL of Water Lysis Solution (WL) (DS0202)** to the Hi-Water Bead tube. Secure the bead tube horizontally on a vortex adaptor. Vortex at maximum speed for 5 minutes.

4) Centrifuge the **Hi-Water Bead Tube (DBCA020)** at 5000 rpm for 1 minute (in a 15 mL rotor). Transfer supernatant to a new 2 mL microcentrifuge tube (provided) (approx. 600 – 650 µL of supernatant can be recovered).

**NOTE:**

- This centrifugation step is optional; if not performed, it will result in minor loss of supernatant.
- The supernatant can be aspirated using a 200µL pipette and placing it at the bottom of the tube to ensure maximum recovery of the lysate.

5) Centrifuge at 13,000 rpm for 1 minute at room temperature (15-25°C). Transfer the supernatant to new 2 mL collection tube (provided) without disturbing pellet.

6) To the above supernatant, add **200 µL of Inhibitor Removal Solution (IRSH) (DS0066)**, vortex it briefly & incubate at 4°C for 5 minutes.

7) Centrifuge the tube at 13,000 rpm for 1 minute at room temperature (15-25°C). Transfer the supernatant to new 2 mL collection tube (provided) without disturbing pellet.

8) Add **650 µL of Binding Solution (WBS) (DS0203)** and mix by vortexing briefly.

9) Load approximately 650 µL of the above solution onto the HiElute Miniprep Spin Column (Capped) and centrifuge for 1 minute at 13,000 rpm at room temperature (15-25°C). Discard the flow-through. Repeat the above step with the remaining sample. Discard the flow-through liquid and reuse the 2.0 mL collection tube (uncapped).

10) Add **650 µl of diluted Wash Solution (WS) (DS0012)** to the column and centrifuge at 13,000 rpm for 1 minute. Discard the flow-through, re-use the same 2.0 mL collection tube with column.

11) Add **650 µl of Wash Solution WT (DS0204)** to the column and centrifuge at 13,000 rpm for 1 minute. Discard the flow-through and centrifuge again at 13,000 rpm for 2 minutes to dry the column.

12) Place the column in a new 2.0 mL collection tube (uncapped) and add **100 µL of Elution Buffer (ET) (DS0040)**. Incubate for 5 minutes at room temperature (15-25°C). Centrifuge at 13,000 rpm for 1 minute. Transfer the eluate to a new tube for DNA storage (Store at -20°C).

**Storage of the eluate:** The eluate contains pure genomic DNA. For short-term storage (24-48 hrs) of the DNA, 2-8°C is recommended. For long-term storage, -20°C or lower temperature (-80°C) is recommended. Avoid repeated freezing and thawing of the sample which may cause denaturing of DNA. The Elution Buffer will help to stabilize the DNA at these temperatures.

## II) Detection of *E. coli O157: H7* by qPCR (Probe based)

Real-time polymerase chain reaction, also called quantitative Polymerase Chain Reaction (qPCR) or kinetic Polymerase Chain Reaction, is a laboratory technique based on the principle of PCR. This technique is used to amplify a targeted RNA sequence by use of hydrolysis probes that are short oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. HiMedia's E. coli O157:H7 Water Detection Kit (Real-Time Probe Based PCR) is designed to detect the outer membrane protein (eae) gene of **E. coli O157:H7 in HEX channel** with **Internal Control (IC) in Texas Red channel**.

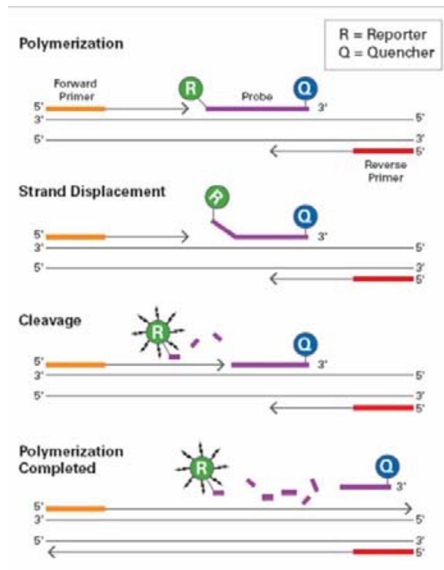
**Positive control**

This is a control reaction using a known template (target pathogen). A positive control is usually used to check that the primers have been designed properly and the PCR conditions have been set up correctly.

**Internal control**

This is a control sequence which is amplified in the same reaction tube along with the target sequence (target pathogen) but detected with a different primer (i.e. Multiplex PCR). An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified.

**Diagrammatic representation of preferential binding of probe specific to DNA fragments in Real-time PCR**




**Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' end of the probe respectively**

**Strand displacement: When the probe is intact, the report dye emission is quenched.**

**Cleavage: During each extension cycle,the DNA polymerae cleaves the reporter dye from the probe**

**Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence**

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). The probes are designed such that they anneal within a DNA region amplified by a specific set of primers. During PCR amplification, these probes will hybridize to the target sequences located in the amplicon i.e. the DNA. As the *Taq* DNA polymerase replicates the template with the bound probe, the 5'- nuclease activity of the polymerase enzyme cleaves the fluorescent probe. The end result in cleavage of the probe is separation of the reporter dye from the quencher dye and increasing the reporter dye signal. As the probe is removed from the target strand, primer extension continues to the end of the template strand. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Thus, inclusion of the probe does not inhibit the overall PCR process.

**Features:**

- Fast and simple
- Good sensitivity and specific results
- Guaranteed reproducible results
- Rapid detection of all relevant clinical pathogens

**General Preparation Instructions:**

- Before use, all PCR components should be completely thawed on ice (4°C).
- Perform the amplification reactions in a clean area, preferably in a biosafety cabinet.
- Use of aerosol barrier pipette tips is recommended to reduce contamination risks from extraneous DNA templates.
- Extract and store positive control sample (if used) separately from all other reagents to avoid contamination and add it to the reaction mix in a separate area.

**A) <u>Procedure for Setting up the PCR Reaction</u>**

The PCR reaction is set up in 0.2 mL PCR tubes. The procedure for setting up the reaction is tabulated below. If about 10 samples have to be tested, the reaction mixture is prepared for 10 samples and their respective template is added in the 0.2 mL tube. A positive and negative control tube is ran in parallel to the sample tubes.

| Components | Product code | DNA Purified from Water Sample (1X) |
|---|---|---|
| Hi-Quanti 2X Realtime PCR Master Mix | MBT180 | 12.5 µL |
| E. coli O157:H7 Primer-Probe Mix | DS0739 | 1 µL |
| Internal Control Primer-Probe Mix | DS0741 | 1 µL |
| Internal Control E DNA | DS0619 | 1 µL |
| Molecular Biology Grade Water for PCR | ML065 | 4.5 µL |
| Template DNA/Negative Control | - | 5 µL |
| Total volume | - | Upto 25 µL |

**NOTE: (Optional) – The user can also set up an additional PCR reaction containing 5 µL Positive Control (DS0353) (as mentioned in the table above) in a separate tube.**

Centrifuge the tube briefly at 6000 rpm for about 10 seconds. Place the tubes in Real-Time PCR machine and set the recommended PCR program (mentioned below). Interpret the data from the amplification plot (observe the Ct values).

**B) Recommended PCR program**

1) Initial denaturation : 95°C for 10 minutes
2) Denaturation : 95°C for 15 seconds
3) Annealing and Extension : 60°C for 30 seconds
   Plate Read : HEX/ Texas Red
4) Hold : 4°C for ∞

(Steps 2–3 including Plate Read) No. of cycles: 40

**C) Quality Control**

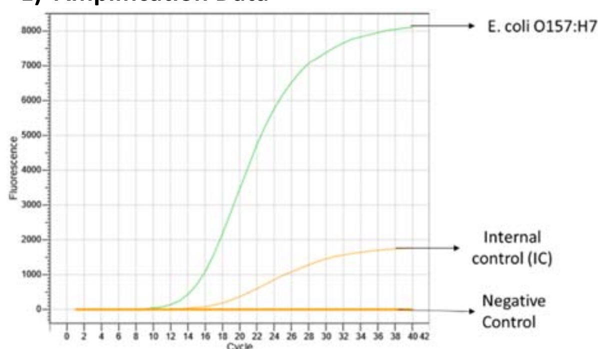
Each lot of HiMedia's E.coli O157:H7 Water Detection Kit (Real time Probe Based PCR) is assayed for contaminating endonuclease, exonuclease and non-specific DNase activities. Functionally tested in DNA amplification.

**D) Data Analysis**

The following conditions should be met for a valid diagnostic test:

| Control | Detection channel | |
|---|---|---|
| | HEX (E. coli O157:H7) | Texas Red (Internal Control) |
| Positive Control | + | + |
| Negative Control | - | + |

**E) Amplification Data**




| Sr. No. | Sample | Ct value |
|---|---|---|
| 1. | E. coli O157:H7 | 11.92 |
| 2. | Internal Control | 16.75 |
| 3. | Negative control | N/A |

**Image representing amplification plot of E. coli O157:H7 DNA with Ct values using HiMedia's E. coli O157:H7 Water Detection Kit (Real-Time Probe Based PCR)**

**F) Data Interpretation**

| Detection Channel | | Result Interpretation |
|---|---|---|
| **HEX (E. coli O157:H7)** | **Texas Red (Internal Control)** | |
| + | +/-* | Positive for E. coli O157:H7 |
| - | + | Negative for E. coli O157:H7 |
| - | - | PCR inhibition or reagent failure.<br>Repeat PCR or repeat extraction from original sample |

*The presence or absence of a signal in the Texas Red channel is not relevant for the validity of the test run due to competition between the test template and Internal Control template.

**Precautions**

Read the procedure carefully before starting the experiment. Wear protective gloves/protective clothing/eye protection/face protection. Follow good clinical laboratory practices while handling clinical samples. Standard precautions should be followed as per established guidelines. Safety guidelines may be referred in safety data sheets of the product.

**Performance and Evaluation**

Each lot of HiMedia's E.coli O157:H7 Water Detection Kit (Real time Probe Based PCR) is tested against predetermined specifications to ensure consistent product quality.

**Troubleshooting Guide:**

| Sr.No. | Problem | Possible Cause | Solution |
|---|---|---|---|
| 1. | HiElute Miniprep Spin Column (Capped) is clogged | Sample volume is large / dense | Use smaller quantity of sample, to salvage the current preparation, clogging can be alleviated by increasing the g force or spinning for longer time until the lysate passes through the spin column. |
| 2. | Poor / Lower yield of genomic DNA | DNA elution is incomplete.<br>Eluate contains residual ethanol from the wash | DNA yield can be improved by incubating the Elution Buffer for 5 minutes at room temperature (15-25°C) after it is added to the column.<br>Ethanol from the final wash should be eliminated completely before eluting DNA. Spin the tubes for longer time to dry the column completely. |
| | | Wash Solution Concentrate was not diluted before use. | Check that the Wash Solution Concentrate is properly diluted with ethanol as per instructions. |
| | | Use of water instead of Elution Buffer for elution of DNA | Elution Buffer is recommended for optimal yields and storage of the genomic DNA. If water is used instead of the Elution Buffer, the pH should be at least 7.0 to avoid acidic conditions which may cause acid hydrolysis of DNA when stored for long periods of time.<br><br>**NOTE:** Only DNase/RNase and Protease free water should be used for eluting DNA |

| | | Background reading is high due to silica fines | The DNA sample can be centrifuged at maximum speed for 1 minute, the supernatant can be used to repeat the absorbance readings. |
|---|---|---|---|
| | | Sample diluted in water | Use either Elution Buffer provided or 10 mM Tris-HCl, 0.5 mM EDTA pH 9.0 or 10 mM Tris-HCl pH 8.0-8.5 as the eluate. |
| | | Improper handling of genomic DNA | All pipetting steps should be executed as gently as possible. Wide orifice pipette tips are recommended to eliminate shearing of the DNA to a large extent. If the isolated DNA is to be used for PCR, mix with gentle pipetting or invert until homogenous, instead of vortexing as it reduces shearing of DNA considerably. |
| 6. | Purity of the DNA is lower than expected; $A_{260}/A_{280}$ ratio is too high. | Traces of ethanol present in the final genomic DNA preparation | After the washing steps, the flow-through should not come in contact with the column. Spin the column for 1 minute at maximum speed (12,000-16,000 x g) if necessary, after emptying the collection tube. |
| 7. | DNA is sheared | Salt is carried over in the final genomic DNA preparation | The column should be transferred to a new capped 2.0 ml collection tube before adding the wash solutions. |
| 8. | No amplification | Degraded samples | Check the integrity of DNA using agarose gel electrophoresis.<br>Use freshly prepared DNA to ensure the availability of intact template sequence for efficient amplification |
| | | Error in protocol setup | Verify that the correct reagent volumes, dilutions and storage conditions have been used. |
| 10. | Variability between replicates | Error in reaction set-up | Prepare a large volume master mix, vortex thoroughly and aliquot into reaction tubes. |
| | | Air bubbles in reaction mix | Briefly centrifuge reaction samples/plate prior to running on a real-time PCR instrument. |
| | | Pipetting error | $C_t$ values of replicates can show increased variation due to poor laboratory technique or imprecise pipettes. |

| 11. | Amplification in negative control | Reagents contaminated | Replace all critical solutions.<br>Repeat the analysis of all tests with fresh aliquots of critical reagents. |
|---|---|---|---|
| 12. | No signal with positive controls | Incorrect programming of the temperature profile of the thermocycler | Compare the temperature profile to the manual. |

**Safety Information**

The E.coli O157:H7 Water Detection Kit (Real time Probe Based PCR) is for laboratory use only, not for drug, household or other uses. The Lysis Solution (C1) contains chaotropic salts, which are irritants. Take appropriate laboratory safety measures and wear gloves when handling. Not compatible with disinfecting agents containing bleach. Please refer the Material Safety Data Sheet (MSDS) for information regarding hazards and safe handling practices.

**Disposal**

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques.

**Technical Assistance**

At HiMedia, we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com.

Storage temperature

Do not use if package is damaged

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Disclaimer :

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