HITACHI
Inspire the Next

# Examples of microbial identification using the DS3000 Compact CE Sequencer


## Summary

The evolutionary history of microorganisms is recorded in ribosomal RNA (rRNA), and a method of phylogenetic classification has been adopted based on this record. In the 18th edition of the Japanese Pharmacopoeia, methods for microbial identification analysis using capillary sequencers are presented. These methods automatically analyze the gene sequence of part of the highly variable region of 16S rRNA for bacteria and the spacer region between 18S rRNA and 5.8S rRNA (ITS1) for fungi, and identify or estimate microorganisms by comparing them with a database.

Here are two examples of identification using the DS3000 Compact CE Sequencer (hereinafter referred to as the DS3000) for microorganisms that exist in our everyday environment. One is the identification of microorganisms from colonies derived from indigenous bacteria to human skin using mannitol salt agar with egg yolk, and the other is the identification of microorganisms from fungi growing on mandarin orange rind. The microorganisms were analyzed using the DS3000. Based on the search database results, the one from human skin was classified into the genus *Staphylococcus*, suggesting the possibility of *Staphylococcus aureus*. The one from the mandarin orange rind was classified in the genus *Penicillium*.


DS3000
Compact CE Sequencer

## Results

Using the Hand Petan Check II containing mannitol salt agar with egg yolk (EIKEN CHEMICAL Co., Ltd.), which can be easily used to test for indigenous bacteria on the hand, the palm was pressed against the medium to form colonies. DNA extracted from the colonies was PCR amplified using the Bacterial 16S rDNA PCR Kit Fast (800) (Takara Bio Inc.), and the cycle sequencing reaction was performed with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The obtained samples were electrophoresed on the DS3000. Forward (F) and reverse (R) 16S rDNA sequences were edited and assembled (Figures 1 and 2) using the DNA sequence analysis software SEQUENCHER, and then searched in the NCBI BLAST® sequence database. The top search result was the genus *Staphylococcus*, so the colony was classified into the genus *Staphylococcus* (Figure 3). Yellowing was also observed in the mannitol salt agar with egg-yolk, suggesting the presence of *Staphylococcus aureus* in the obtained colonies. Therefore, both the sequence database search results and the yellowing in the mannitol salt agar with egg-yolk indicated the possibility of *Staphylococcus aureus*.

In addition, a colony was isolated from mold (fungi) growing on mandarin orange rind using potato dextrose agar, and the extracted DNA was PCR amplified with Fungal rDNA (ITS1) PCR Kit Fast and Fungal rDNA (D1/D2) PCR Kit Fast (Takara Bio Inc.), and a cycle sequencing reaction was performed as described above. The obtained samples were electrophoresed using the DS3000. F and R sequences of the ITS1 region and the D1/D2 region were edited and assembled (Figure 4) using the DNA sequence analysis software SEQUENCHER, and then searched in the NCBI BLAST® sequence database. In both regions, the top search result was the genus *Penicillium*, so the colony was presumed to be from the genus *Penicillium* (Figures 5 and 6).

*Staphylococcus aureus*, which was obtained from the Hand Petan Check II containing mannitol salt agar with egg-yolk medium, is said to be present in the nasal cavity of approximately 20% of healthy adults (Reference 1). Thus, detection of this bacterium from the hand is valid. It is also reasonable that the genus *Penicillium* was detected in the mold on the mandarin orange rind.

## Conclusion

It was confirmed that the DS3000 can be used to estimate bacteria and fungi by referring to the microbial identification analysis method described in the 18th edition of the Japanese Pharmacopoeia. The DS3000 can also be used to identify bacteria and fungi gene sequences.

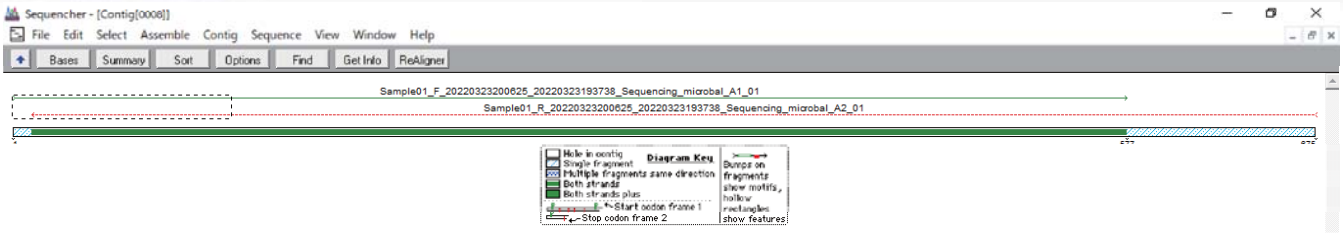


**Figure 1: Assembly overview of Experiment 1 using SEQUENCHER**


**Figure 2: Assembly and peak shapes (around 330 bp) for Experiment 1 using SEQUENCHER**

| Subject accession | Discription | e-value | bitscore | Percentage of identical matches | Alignment length |
|---|---|---|---|---|---|
| NR_113345.1 | Staphylococcus haemolyticus strain JCM 2416 16S ribosomal RNA | 0 | 1243 | 99.852 | 675 |
| NR_036955.1 | Staphylococcus haemolyticus strain SM 131 16S ribosomal RNA | 0 | 1238 | 99.704 | 675 |
| NR_116627.1 | Staphylococcus devriesei strain KS-SP 60 16S ribosomal RNA | 0 | 1227 | 99.407 | 675 |
| NR_036956.1 | Staphylococcus hominis strain DM 122 16S ribosomal RNA | 0 | 1216 | 99.111 | 675 |
| NR_136463.1 | Staphylococcus petrasii subsp. pragensis strain CCM 8529 16S ribosomal RNA | 0 | 1206 | 98.815 | 675 |
| NR_118248.1 | Staphylococcus petrasii subsp. jettensis strain SEQ110 16S ribosomal RNA | 0 | 1206 | 98.815 | 675 |
| NR_118450.1 | Staphylococcus petrasii strain CCM 8418 16S ribosomal RNA | 0 | 1205 | 98.815 | 675 |
| NR_041323.1 | Staphylococcus hominis subsp. novobiosepticus strain GTC 1228 16S ribosomal RNA | 0 | 1205 | 98.815 | 675 |
| NR_132590.1 | Staphylococcus petrasii subsp. croceilyticus strain MCC10046 16S ribosomal RNA | 0 | 1201 | 98.667 | 675 |

**Figure 3: List of NCBI BLAST® search results for Experiment 1**


**Figure 4: Assembly and peak shapes (D1/D2, around 320 bp) for Experiment 2 using SEQUENCHER**

| Subject accession | Discription | e-value | bitscore | Percentage of identical matches | Alignment length |
|---|---|---|---|---|---|
| NR_171619.1 | Penicillium hordei CBS 701.68 ITS region; from TYPE material | 3.43E-125 | 444 | 97.328 | 262 |
| NR_163669.1 | Penicillium fuscoglaucum CBS 261.29 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_163549.1 | Penicillium neoechinulatum CBS 169.87 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_163544.1 | Penicillium hirsutum CBS 135.41 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_163541.1 | Penicillium lanosocoeruleum CBS 215.30 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_172035.1 | Penicillium speluncae DAOMC 251701 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_138261.1 | Penicillium molle CBS 456.72 ITS region; from TYPE material | 1.6E-123 | 438 | 96.947 | 262 |
| NR_163685.1 | Penicillium caseifulvum CBS 101134 ITS region; from TYPE material | 5.74E-123 | 436 | 96.935 | 261 |
| NR_163684.1 | Penicillium cavernicola CBS 100540 ITS region; from TYPE material | 2.06E-122 | 435 | 96.923 | 260 |

**Figure 5: List of NCBI BLAST® search results for Experiment 2 (ITS1)**

| Subject accession | Discription | e-value | bitscore | Percentage of identical matches | Alignment length |
|---|---|---|---|---|---|
| NG_069698.1 | Penicillium kewense CBS 344.61 28S rRNA gene | 0 | 1110 | 99.671 | 607 |
| NG_069786.1 | Penicillium sinaicum CBS 279.82 28S rRNA gene | 0 | 1109 | 99.67 | 606 |
| NG_068959.1 | Penicillium molle CBS 456.72 28S rRNA gene | 0 | 1109 | 99.67 | 606 |
| NG_069811.1 | Penicillium neoechinulatum CBS 169.87 28S rRNA gene | 0 | 1105 | 99.506 | 607 |
| NG_069644.1 | Penicillium cyclopium CBS 144.45 28S rRNA gene | 0 | 1105 | 99.506 | 607 |
| NG_069854.1 | Penicillium sclerotigenum CBS 101033 28S rRNA gene | 0 | 1103 | 99.505 | 606 |
| NG_069803.1 | Penicillium freii CBS 476.84 28S rRNA gene | 0 | 1103 | 99.505 | 606 |
| NG_069626.1 | Penicillium egyptiacum CBS 244.32 28S rRNA gene | 0 | 1101 | 99.504 | 605 |
| NG_069855.1 | Penicillium caseifulvum CBS 101134 28S rRNA gene | 0 | 1099 | 99.341 | 607 |

**Figure 6: List of NCBI BLAST® search results for Experiment 2 (D1/D2)**

## Experiment 1: Identification of microorganisms from bacterial colonies indigenous to human skin

### 1. Culture

A medium for environmental microorganism tests, the Hand Petan Check II containing mannitol salt agar with egg yolk (EIKEN CHEMICAL Co., Ltd.), was used (Figure 7). Mannitol salt agar with egg yolk is a selective isolation medium for staphylococci that can simultaneously detect yolk reaction and mannitol degradability. The growth of bacteria other than *Staphylococcus aureus* is inhibited by the high concentration of salt added. When *Staphylococcus aureus* is present and decomposes mannitol, the culture medium becomes acidic and the phenol red, an indicator, turns yellow. When *Staphylococcus aureus* does not decompose, the culture medium becomes alkaline and shows either no discoloration or becomes pink. Furthermore, the lecitho-vitellin reaction can be observed by adding egg yolk solution. A light yellow-milky white opaque band appears around colonies of bacteria that are positive for the lecitho-vitellin reaction.

After pressing a hand onto the surface of the medium (Figure 7), the medium was incubated at 28˚C for 3 days. Multiple bacterial colonies formed and the agar medium around the colonies turned yellow (Figure 8).

### 2. DNA extraction

Seven of the colonies formed on the agar medium were selected (Figure 8), and bacterial DNA was extracted using MightyPrep reagent for DNA (Takara Bio Inc.) (Reference 2; all references to reagents are the manufacturer-provided protocols). First, the bacteria were removed from the colonies on the agar medium with a sterile toothpick and suspended in 100 μl of MightyPrep reagent for DNA. Next, using a heat block, they were heated at 95˚C for 10 minutes. Finally, they were centrifuged at 12,000 to 15,000 rpm for 2 minutes. The supernatant was used as a PCR template.

### 3. DNA amplification and purification

The DNA obtained in step 2 was PCR-amplified for specific regions within the bacterial 16S rDNA region using the Bacterial 16S rDNA PCR Kit Fast (800) (Takara Bio Inc.) (Table 1 and 2, and Reference 3). The obtained PCR amplification product was purified using NucleoSpin®-Gel-and-PCR-Clean-UP (MACHEREY-NAGEL®) (Reference 4). DNA elution from the column was performed using 20 μl of Elution Buffer.

### 4. Cycle sequencing reaction and purification

Cycle sequencing reactions were performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) (Tables 3 and 4, and Reference 5). They were then prepared using the BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific) (Table 5 and Reference 6), stirred for 20 minutes under conditions of 2,000 rpm or more and amplitude of 4 mm or more, and purified.

### 5. Capillary electrophoresis

Electrophoresis was performed using the module for the BigDye XTerminator™ Purification Kit (Table 6).

### 6. Analysis

A BLASTN search was performed using contigs created from the F and R sequences. The latest version (as of March 2022) of NCBI 16S ribosomal RNA (Bacteria and Archaea type strains) [ftp://ftp.ncbi.nlm.nih.gov/blast/db/16S_ribosomal_RNA.tar.gz] was used as the sequence database.

**Figure 7: Culture medium for the environmental microorganism test, Hand Petan Check II, mannitol salt agar with egg yolk (EIKEN CHEMICAL Co., Ltd.)**

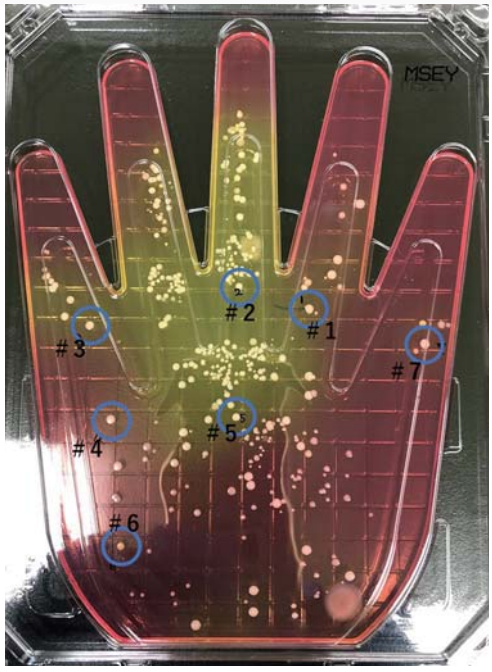


**Figure 8: Result of incubation (28˚C) for 3 days after pressing the hand against the mannitol salt agar with egg yolk**

Numbers indicate colonies used in this analysis.

**Table 1: PCR mix for the Bacterial 16S rDNA PCR Kit Fast (800)**

| Reagent | Volume (μL) |
|---|---|
| TaKaRa® Taq HS Fast Detect Premix (2×) | 12.5 |
| 16S rDNA Primer Mix (bacteria) (10×) | 2.5 |
| MightyPrep reagent for DNA extraction | 2.5 |
| Nuclease Free Water | 7.5 |
| Total reaction volume | 25 |

**Table 2: PCR conditions for the Bacterial 16S rDNA PCR Kit Fast (800)**

| Temperature (˚C) | Time (s) | Cycle |
|---|---|---|
| 92 | 60 | ×1 |
| 92 | 5 | ×25 |
| 50 | 1 | |
| 68 | 8 | |
| 72 | 60 | ×1 |
| 4 | hold | |

**Table 3: Cycle sequencing mix for the BigDye™ Terminator v3.1 Cycle Sequencing Kit**

| Reagent | Volume (μL) |
|---|---|
| BigDye™ Terminator v3.1 | 1 |
| Template (PCR products after column purification)* | X |
| Primer (1 pmol/μl) | 1 |
| BigDye™ v3.1 Cycle Sequencing Kit 5×Sequencing Buffer | 1.75 |
| Deionized Water | up to total Volume |
| Total reaction volume | 26 |

*The quantities of DNA template were measured using a Qubit™ 4 Fluorometer (Thermo Fisher Scientific) and templates were prepared to a final volume of 10 to 40 ng.

**Table 4: Cycle sequencing conditions for the BigDye™ Terminator v3.1 Cycle Sequencing Kit**

| Temperature (°C) | Time (s) | Cycle |
|---|---|---|
| 96 | 60 | ×1 |
| 96 | 10 | ×25 |
| 50 | 5 | |
| 60 | 240 | |
| 4 | hold | |

**Table 5: Purification mix for the BigDye Xterminator™ Purification Kit**

| Reagent | Volume (μL) |
|---|---|
| Cycle sequence reacted sample | 10 |
| SAM™ Solution | 45 |
| BigDye Xterminator™ Solution | 10 |
| Total reaction volume | 65 |

**Table 6: Electrophoresis conditions**

| | |
|---|---|
| Polymer | Polymer7 |
| Dye Set | AB 4-dye sequencing |
| Matrix Standard | 31xx Matrix Standards Kit, BigDye™ Terminator v3.1 (Thermo Fisher Scientific) |
| Assay | AB_Xseq_36_Fast |
| Injection Voltage/Time | 1.2 kV/4 s |
| Run Voltage | 14 kV |
| Run Time | 900 s |

## Experiment 2: Identification of microorganisms from fungi growing on mandarin orange rind

In addition to the ITS1 region (150 to 500 bp) between 18S rRNA and 5.8S rRNA, the D1/D2 region within the 28S rDNA region (approximately 0.6 kb) was also set as the target.

**1. Culture**

Colonies were isolated from mold (fungi) (Figure 9) growing on the rind of a mandarin orange using potato dextrose agar (PDA).

**2. DNA extraction**

Fungal DNA was extracted from colonies formed on the agar medium (Figure 10) using the MightyPrep reagent for DNA (Takara Bio Inc.). The procedure for using the MightyPrep reagent for DNA is the same as in Experiment 1.

**3. DNA amplification and purification**

The DNA obtained in step 2 was PCR-amplified for the ITS1 region using the Fungal rDNA (ITS1) PCR Kit Fast (RR183 A) (Takara Bio Inc.) (Tables 7 and 8, and Reference 7). Also, the D1/D2 region was PCR-amplified using the Fungal rDNA (D1/D2) PCR Kit Fast (RR184 A) (Takara Bio Inc.) (Tables 9 and 10, and Reference 8). The obtained PCR amplification product was purified using NucleoSpin® -Gel-and-PCR-Clean-UP (MACHEREY-NAGEL®). DNA elution from the column was performed using 20 $\mu$l of Elution Buffer. Figure 11 shows the results of gel electrophoresis.

**4. Cycle sequencing reaction and purification**

Similar to Experiment 1, cycle sequencing reactions were performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) (Tables 3 and 4). They were then prepared using the BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific) (Table 5), stirred for 20 minutes under conditions of 2,000 rpm or more and amplitude of 4 mm or more, and purified.

**5. Capillary electrophoresis**

Electrophoresis was performed using the module for the BigDye XTerminator™ Purification Kit (Table 6).

**6. Analysis**

A BLASTN search was performed using contigs created from the F and R sequences. For the sequence database targeting the ITS1 region, the NCBI Internal transcribed spacer region (ITS) from Fungi type and reference material [ftp://ftp.ncbi.nlm.nih.gov/blast/db/ITS_RefSeq_Fungi.tar.gz] was used. For the sequence database targeting the D1/D2 region, the NCBI 28S ribosomal RNA sequences (LSU) from Fungi type and reference material [ftp://ftp.ncbi.nlm.nih.gov/blast/db/28S_fungal_sequences.tar.gz] were used.

**Figure 9: Mold (fungi) growing on mandarin orange rind**

**Figure 10: Fungal colonies from mandarin orange rind isolated on potato dextrose agar (PDA)**

**Table 7: PCR mix for the Fungal rDNA (ITS1) PCR Kit Fast**

| Reagent | Volume ($\mu$L) |
|---|---|
| TaKaRa® Taq HS Fast Detect Premix (2×) | 12.5 |
| rDNA ITS1 Primer Mix (10×) | 2.5 |
| MightyPrep reagent for DNA extraction | 2.5 |
| Nuclease Free Water | 7.5 |
| Total reaction volume | 25 |

**Table 8: PCR conditions for the Fungal rDNA (ITS1) PCR Kit Fast**

| Temperature (˚C) | Time (s) | Cycle |
|---|---|---|
| 92 | 60 | ×1 |
| 92 | 5 | ×30 |
| 50 | 1 | |
| 68 | 6 | |
| 68 | 60 | ×1 |
| 4 | hold | |

**Table 9: PCR mix for the Fungal rDNA (D1/D2) PCR Kit Fast**

| Reagent | Volume (μL) |
|---|---|
| TaKaRa® Taq HS Fast Detect Premix (2×) | 12.5 |
| rDNA D1/D2 Primer Mix (10×) | 2.5 |
| MightyPrep reagent for DNA extraction | 2.5 |
| Nuclease Free Water | 7.5 |
| Total reaction volume | 25 |

**Table 10: PCR conditions for the Fungal rDNA (D1/D2) PCR Kit Fast**

| Temperature (˚C) | Time (s) | Cycle |
|---|---|---|
| 92 | 60 | ×1 |
| 92 | 5 | ×30 |
| 55 | 1 | |
| 68 | 6 | |
| 68 | 60 | ×1 |
| 4 | hold | |

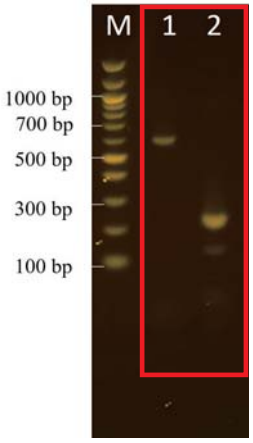


**Figure 11: Gel electropherogram of ITS and D1/D2 regions of fungi from mandarin orange rind**

| Symbol in figure | Gel electrophoresis sample |
|---|---|
| M | 100 bp Ladder (Marker) |
| 1 | D1/D2 |
| 2 | ITS1 |

References
(1) Japanese Society for Bacteriology *Staphylococcus* https://jsbac.org/youkoso/staphylococcus.html
(2) MightyPrep reagent for DNA Product Manual v201509Da
(3) Bacterial 16S rDNA PCR Kit Fast (800) Product Manual v202203Da (Japanese only)
(4) NucleoSpin®-Gel-and-PCR-Clean-UP User manual March 2023 / Rev.08
(5) BigDye™ Terminator v3.1 Cycle Sequencing Kit USER GUIDE 4337035 Rev.D
(6) BigDye XTerminator™ Purification Kit USER GUIDE 4374408 Rev.D
(7) Fungal rDNA (ITS1) PCR Kit Fast Product Manual v202203Da (Japanese only)
(8) Fungal rDNA (D1/D2) PCR Kit Fast Product Manual v201802Da (Japanese only)

## Main specifications for the DS3000

### ■ Main unit specifications

| Item | Details |
|---|---|
| Number of capillaries | 4 |
| Capillary length | 36 cm |
| Sample format | 8-tube strip × 4 |
| Device control | Touch panel PC |
| Number of Dyes | 6 |
| Application | Sequencing analysis / Fragment analysis |
| Size | 400 (W) × 600 (D) × 600 (H) mm |
| Weight | 45 kg |
| Performance guarantee temperature | 15 – 30˚C |
| Performance guarantee humidity | 20 – 80% RH (no condensation) |
| Power input | 100 – 240 ±10% VAC, 50/60 Hz |
| Rated power | 260 VA |
| Supported secondary analysis software | • Mutation Surveyor (SoftGenetics, LLC, sold separately)<br>• GeneMarker (SoftGenetics, LLC, sold separately)<br>• GeneMarker HID (SoftGenetics, LLC, sold separately) |

### ■ Run module specifications

| Run Module | Application | Polymer type | Contiguous Read Length*1 (bp, QV20 CRL) | Average run time (minutes) |
|---|---|---|---|---|
| Fast_Sequence36_Polymer7 | Sequencing analysis | Polymer7 | ≥600 | ≤32 |
| Standard_Sequence36_Polymer7 | Sequencing analysis | Polymer7 | ≥700 | ≤60 |
| BDx_Fast_Sequence36_Polymer7 | BDx sequencing analysis | Polymer7 | ≥600 | ≤32 |
| BDx_Standard_Sequence36_Polymer7 | BDx sequencing analysis | Polymer7 | ≥700 | ≤60 |

| Run Module | Application | Polymer type | Average run time (minutes) | Sizing precision*2 (bp, 50 – 400 bp) |
|---|---|---|---|---|
| Fragment_Analysis36_Polymer7 | Fragment analysis | Polymer7 | ≤35 | NA |
| Fragment_Analysis36_Polymer4 | Fragment analysis | Polymer4 | ≤44 | <0.16 |

*1 Contiguous Read Length (bp, QV20 CRL) is measured with BigDye™ Terminator v3.1. Sequencing Standard Kit (Thermo Fisher Scientific, sold separately)

*2 Sizing precision (bp, 50 - 400 bp) is measured with PowerPlex™ ESI17 Fast Allelic Ladder and WEN ILS 500 ESS. (Promega®, sold separately)

· BigDye™ is a registered trademark of Thermo Fisher Scientific Inc.

· Promega®, PowerPlex™ is a registered trademark of Promega Corporation.

### ■ Consumables specifications

| Product name | Part number | Details | Maximum number of uses | Remarks |
|---|---|---|---|---|
| Capillary Cartridge 36 cm | 613-0330 | 1 pcs | 300 injections/unit | Storage temperature: 15 – 30˚C |
| Buffer | 613-0252 | Anode Buffer × 2 cartridges<br>Cathode Buffer × 2 cartridges | 80 injections/unit | Storage temperature: 2 – 10˚C |
| Polymer7 | 613-0251 | 4 cartridges | 16 injections/unit | Storage temperature: 2 – 10˚C |
| | 613-0291 | 4 cartridges | 24 injections/unit | Storage temperature: 2 – 10˚C |
| Polymer4 | 613-0250 | 4 cartridges | 16 injections/unit | |
| | 613-0290 | 4 cartridges | 24 injections/unit | |
| Septa for Cathode Buffer Cartridge | 613-7231 | 10 pcs | Single-use | |
| Retainer for Cathode Buffer Cartridge | 613-7233 | 4 pcs | – | |
| Septa for 8 well tubes | 613-7230 | 24 pcs | Single-use | |
| Base and Retainer for 8 well tubes | 613-7232 | 4 pcs | – | |
| Anode Electrode Assembly | 613-7263 | 1 pc | – | |

Specifications in this catalog are subject to change with or without notice,
as Hitachi High-Tech Corporation continues to develop the latest technologies and product for its customers.

CAUTION:For correct operation, follow the instruction manual when using the instrument.

NOTICE:The system is For Research Use Only, and is not intended for any animal or human therapeutic or diagnostic use.
Hitachi High-Tech does not guarantee the performance on the document with every possible sample under every possible condition.


**Hitachi High-Tech Corporation**

**Tokyo, Japan**
**www.hitachi-hightech.com/global/science/**
Toranomon Hills Business Tower, 1-17-1 Toranomon, Minato-ku,
Tokyo 105-6409, Japan
customercenter.ev@hitachi-hightech.com



**HTB-DS-E016** 2024.1