Educational Support to Agrarian and Agri-Food University Programmes in Mongolia

Laboratory Handbook

Microbiology and Molecular Biology Methods for Identification of Foodborne Bacteria

Written by

Dr. Khulan Janchiv (NUM) and Dr. Dulguun Dorjgotov (MUST)

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Alkaline protocol for genomic DNA isolation from E.coli bacteria (mini genomic prep)

In order to extract DNA, the cell wall and cell membrane must first be broken down. It is also necessary to inactivate protein molecules, as they may contain DNAase (DNase refers to all types of DNA-breaking enzymes) enzymes, which degrade DNA (break it down by nucleotides). Saturated phenol and chloroform compounds are widely used in practice to break down and isolate proteins from DNA. Finally, it must be separated from the RNA. Because DNA and RNA are so chemically close, they are difficult to separate by simple physicochemical methods. RNA contamination can adversely affect PCR.

The following reagents and tools are required to extract genome DNA. These include: Lysogeny broth, E.coli DH5α cells, reagents, TE (Tris-EDTA) buffer (pH 8.0), 10% SDS (sodium dodecylsulphate), proteinase K enzyme, phenol-chloroform mixture (1: 1).) solution, 5M sodium acetate (pH5.2), 100% isopropanol, 70% ethanol, sterile distilled water, 2 ml eppendorf tube, automatic µ-pipette, µ-nozzle, µ-centrifuge.Урвалжуудыг дараах байдлаар бэлтгэж туршилтад ашиглана. Including:

1. TE buffer (pH 8.0) - 10 µM Tris (pH 8.0 pH adjust with (HCl)), and 1 DM EDTA (pH 8.0 pH adjust with (NaOH)), dry NaOH to dissolve EDTA . EDTA is soluble only in alkaline media.

2. 10% SDS - Dissolve 10 g of dodecyl sulfate (SDS) in 100 ml of sterile distilled water. Do not inhale SDS in dry areas. If the room temperature is below 10 ° C, the SDS will precipitate again.

3. Proteinase K solution - Dissolve 10 g of proteinase K in 1 ml of sterile distilled water.

4. Phenol-chloroform mixed solution - pH plays an important role in the preparation of this solution. DNA clearance requires a pH=7-8, and these nucleic acids are retained in the aqueous phase. If phenol is acidic, the DNA is shift to the organic phase. Instead, acidic pH = 4 phenol is used to purify RNA. Mix equal amounts of phenol and chloroform and store the mixture on ice. Add 20 ml of TE buffer and shake for 15 min. The excess dirt on

the surface is then removed with a suction pipe. Repeat this process 4-5 times. Finally, add 30-40 ml of TE buffer to the mixture and store on ice.

5. 5M sodium acetate (pH5.2) - Dissolve 41 g of sodium acetate in 100 ml of distilled water and adjust the pH with acetic acid.

Sequence of work to be performed:

- for 16 to 18 hourscultured bacterial culture transfer to 2 ml eppendorf tube and centrifuge the cells for about 10 min.
- Remove the supernatant, add 875 µL of TE buffer to the pellet and prepare a soft mixture suspension.
- Then add 100 µL of 10% SDS and 5 µL of Proteinase K solution to the cell.
- Mix the mixture gently and incubate at 37 ° C for about 1 hour. Mix again every 10 minutes.
- Then add 1 ml of the phenol-chloroform mixture to the above concentration and mix well with vortex. Perform this step on the laminar box.
- centrifuge mixture at room temperature for 10 min at a speed of 10,000 rpm.
- After centrifugation, three phases are formed in the eppendorf tube. These are: the aqueous phase at the top, the intermediate phase with a very thin precipitate in the middle, and the organic phase with phenol / chloroform at the bottom. Pipette the aqueous phase or liquid part of the intermediate phase without disturbing it and transfer it to a new tube.
- Then add the same volume of chloroform mixture, re-centrifuge the liquid portion and collect it in a new tube.
- Add 100 ml of 5M sodium acetate and mix gently.
- Add 1 ml of isoproponal and mix vigorously until the DNA precipitates.
- The above concentration is then centrifuged at 5000 rpm for 10 minutes.
- Remove the liquid and add 1 ml of 70% cold ethanol (70% ethanol stored freezer)
- The above mixture is centrifuged at 5000 rpm for 10 minutes.
- After air drying for 5 minutes, add 200 µL of TE buffer or distilled water.

- 10 µL of DNA sample can be used further and diluted with 1-2 ml of distilled water.

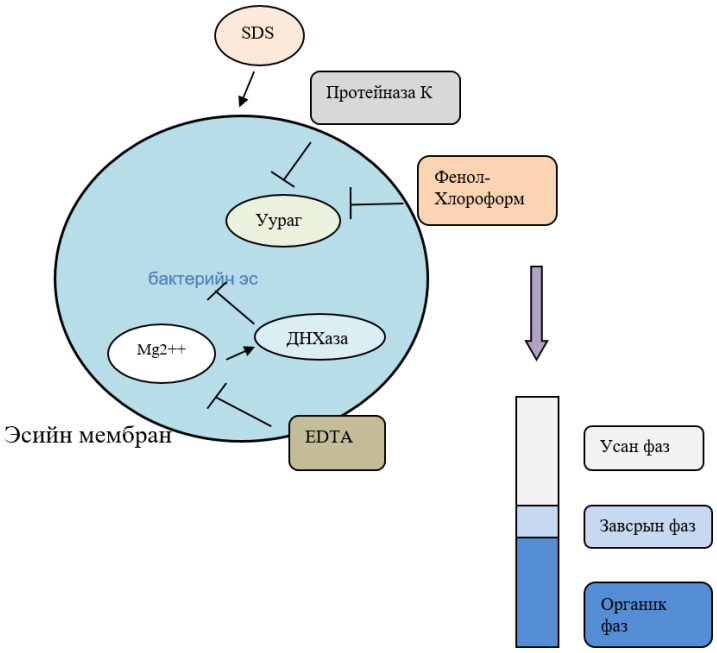
- DNA concentration is determined using a quartz cuvette on a 260 nm spectrophotometer.

Things to keep in mind when performing work:

- Phenol is a very strong acid, so it is dangerous to burn, and chloroform is a carcinogen, so it is necessary to follow safety rules.

- Depending on the source of the DNA, the reaction time may be extended after the addition of Proteinase K solution.

- Depending on the source of the DNA, the process of adding a phenol-chloroform mixture solution is repeated several times to obtain pure DNA.



DNA separation scheme from E.coli cells

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DNA testing quality protocol

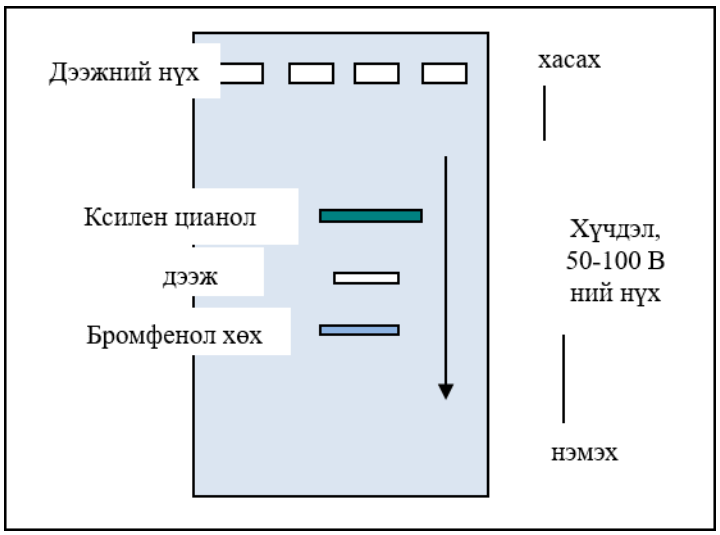
This test is an inexpensive and effective way to isolate DNA molecules and determine their visibility using agarose gel electrophoresis. Agarose gel electrophoresis is widely used to separate biologically negatively charged molecules such as DNA, RNA, and protein molecules (which become negatively charged at pH 9). Agarose is a polysaccharide polymer compound composed of D and L-galactose bound by α and β bonds and is a protein with a molecular weight not exceeding 200 Kda and Distinguish between RNA and DNA consisting of 50or more bases.Agarose is a white compound that dissolves easily in boiling water and turns into a gel when cooled.

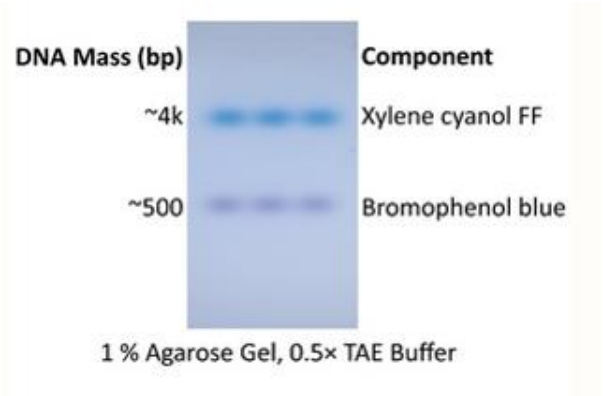
DNA and RNA are negatively charged molecules, and this method is based on the fact that during electrophoresis, the molecules move to the plus or anode poles and are located on the gel according to their weight.

The speed at which DNA travels to the anode depends on the size of the gel well, the voltage, the molecular size, the composition of the gel, the state, and the condition of the buffer solution.Agarose gel is used to separate DNA molecules from 0.7% (to separate large weight DNA based on 5-10 kb) and 2% (to separate small weight DNA based on 0.2-1 kb).

After gel electrophoresis, the DNA molecule cannot be seen with the naked eye, so ethydium bromide (EtBr) is added to the gel as a chromogen. This substance is located between the strands of DNA and emits a yellow light under the influence of ultraviolet light.

During electrophoresis, an loading dye buffer solution containing a dye is prepared, which is important in controlling the transition process in the gel during electrophoresis. Bromphenol blue and xylene cyanol are used as dyes, while glycerol or sucrose are used as loaders. Xylene cyanol forms a bluish-green color and bromphenol forms a bluish circle. If the gel electrophoresis is successful, the two colors on the gel will be clearly visible.





Loading buffer transition on gel.

The test requires the following reagents, substances, and instruments. These include:

- Electrophoresis buffer: 1 x TAE (Tris base, acetic acid, EDTA mixture) buffer
- Pure agarose
- Electrophoresis tank, gel tray, gel comb, voltage, plastic insulating tape, electrode
- Ethidium bromide: 10 μg / ml
- 5 x Gel loading dyes
- DNA marker solution, DNA sample, gloves and pipette

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Sequence of work to be performed:

1. Prepare a 1% agarose gel

- Dissolve 0.5 g of agarose in 50 ml of 1 x TAE buffer. (It is recommended to use a 250 ml flask)
- Heat the agarose solution for about 1 minute. Laboratory microwave ovens may be used.
- After cooling, add 2 µl of ethidium bromide and mix gently.
- Then carefully pour the cooled solution into the gel tray. Before pouring, the gel comb should be soaked in a gel dish.
- Wait until the solution freezes and forms a gel.

2. Load the sample

- Carefully pour the tray gel solution into the electrophoresis tank. At this point, the tank is filled with 1x TAE buffer solution.
- Prepare the sample. Including: 8 µl (0.008 ml) DNA sample 2 µl (0.002 ml) 5x gel loading dye
- Remove the comb from the gel and pipette the sample into the well.
- Plug in the electrode and charge at 50-100 volts for about 20 minutes.
- To monitor the process on the gel, observe the color of the bromophenol blue dye and stop the electrophoresis if the marker ran up to three-quarters of the gel.
- After electrophoresis, remove the gel tray from the tank and collect the instruments.

3. Calculate the results

- Place the gel on a UV transilluminator and note the orange spots on the gel.

Things to consider when doing the work:

- Always wear gloves when handling ethidium bromide
- Be careful and cautious as the gel may break
- Wear laboratory glasses when working with ultraviolet light

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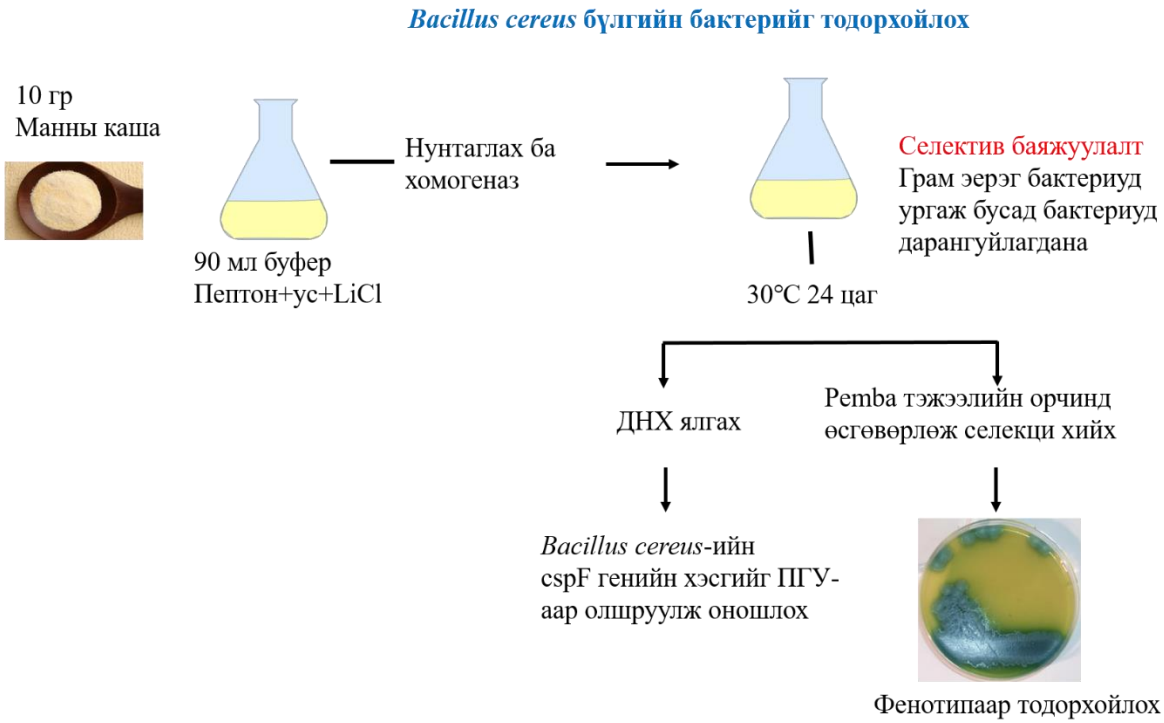

PCR diagnosis of *Bacillus cereus* bacteria

Methodology based on research by Francis et al (1998). The following primers can be used to amplify 284 bp of the cspF gene of the *Bacillus cereus* by PCR:

Primer name	Primer sequences 5’ 3’
BcAPR1	CCT(C/T)TTGGCCTTCTTCTAA
BcFF2	GAGATTTAAATGAGCTGTAA

“Discrimination of Psychrotrophic and Mesophilic Strains of the Bacillus cereus Group by PCR Targeting of Major Cold Shock Protein Genes” Francis K.P., Von Stetten F., Stewart G.S., and Siegfried S, (1998). Applied and Environmental Microbiology. 64. (9), 1178-1183.

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DNA sequencing procedure:

- Transfer 5 ml of bacterial culture to a 15 ml tube with 300 µl Chelex balls (prepared in 25% sterile miliQ water).
- Vortexed and centrifuged at 7000 rpm for 7 minutes
- Pour the liquid on top (supernatant).
- Dissolve 200 µl of precipitate in distilled water
- Boil for 100 ℃ 10 min to lysis the cells.
- Centrifuge at 4 ℃ for 7 min 7000 rpm
- Gently mix the supernatant (100 µl is sufficient and keep on ice). Dilute 1 aliquot 10 times in 50 µl of distilled water. The final volume is 50 µl. DNA concentrations can be measured on a spectrophotometer or NanoDrop.

The sequence of work to be performed by PCR amplification of the cspF-specific sequence:

Place E tube on ice and prepare a PCR master mix.

Reagent	First	Last	Vol/reagemt	Vol/4 reagent
Buffer	10X	1X	2.5 µl	10 µl
dNTPs	10 mM	0.2 mM	0.5 µl	2 µl
Primer BcAPR1	10 µM	0.8 µM	2 µl	8 µl
Primer BcAFF2	10 µM	0.8 µM	2 µl	8 µl
ddH_2O			11.8 µl	4.5 µl
Taq polymerase	5000 U/mL	1U	0.2 µl	0.8 µl

- VortexMaster mix
- Centrifuge for a short time
- 20 µl PCR tube gas (on ice)

Add a DNA sample
- Add 5 µl DNA samples to the first PCR tube
- Add positive control to tube 2

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- Tube 3 has negative control and distilled water
- Centrifuge 3 tubes in a short time

Things to keep in mind when performing work:
All operations are performed on ice.
PCR
Francis et al (1998) stabilized PCR methodology:

Cycle	Temperature	Time
1 cycle	95 ℃	5 min
30 cycles	95 ℃	15 sec
	50 ℃	30 sec
	72 ℃	30 sec
1 cycle	72 ℃	7 min

Gel electrophoresis:

Run the PCR product in 2% agarose gel containing EtBr in the TBE buffer at 80V for 1.5 h.
Preparation of agarose gel
- Prepare 300 ml of 1X TBE
- Take 40 ml of 1X TBE and prepare a 2% (w / v) agarose gel. Melt, cool to 55 ℃ and add EtBr.
- Put the comb in the gel container and freeze the gel with EtBr
- Place the gel in the electrophoresis chamber, pour in the remaining 260 ml of buffer and then remove the comb.

Running gel
- Add 2 µl of 6X gel loading dye to 10 µl PCR product and mix
- Pour the mixture into the gelwell: well 1 is a 5 µl marker, well 2 is a PCR sample, well 3 is positive and well 4 is negative.
- Run with TBE buffer at 80V for 1.5 hours

Results of gel electrophoresis

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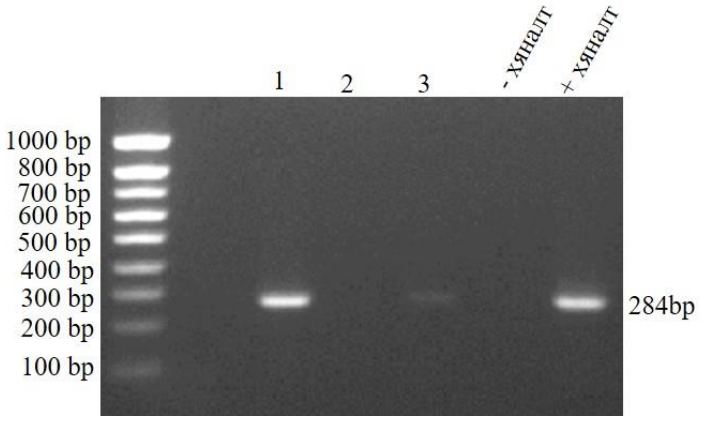

Зураг дээр 284 bp бүхий ПГУ-ын бүтээгдэхүүнийг харуулсан байна.

Pemba nutrient medium

BACILLUS CEREUS SELECTIVE AGAR BASE

Cat.no.G156

Selective and diagnostic (pure culture) culture medium for *Bacillus cereus* infected with various foods.

The Pemba culture medium is a medium designed to detect small amounts of *B. cereus* in other contaminated specimens. Bacillus cereus is a specific secretory agar that is able to differentiate only *B. Cereus* from other bacilli by its ability to tolerate polymyxin, the absence of mannitol fermentation, and its ability to contain lecithinase.
Bacillus cereus is found in raw and processed foods and causes gastrointestinal diseases. *B. Cereus* is widely reported to be transmitted from rice, boiled meat, and cooked foods. Hardy Diagnostics Bacillus cereus Selective agar is:

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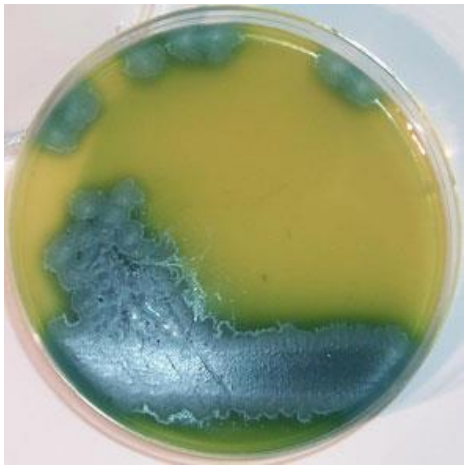

- Contains nutrients such as nitrogen, carbohydrates and meat peptone.
- Maintain NaCl osmosis balance.
- Pyruvate sodium increases the formation of spores and precipitates egg yolks.
- Magnesium sulfate acts as a divalent cation in the growth factor of *Bacillus spp*.
- Mannitol fermentation is detected by a bromothymol blue pH indicator, which results in clones of Bacillus cereus becoming silvery blue.
- Egg yolk suspension is used as a source of lecithin for lecithinase reactions.
- Polymyxin B was added to inhibit gram-positive bacteria.

A clone of *B. Cereus* is wavy, 5 mm in diameter, blue in color, and the surrounding sediment is the same color. This distinguishes *B. Cereus* from allbacilliexcept *B. Thuringiensis. Staphylococcus aureus, Serratia marcescens,* and *Proteus vulgaris,* which grow on egg yolks, differ from *B. Cereus* in the morphology and color of the colony. *B. Cereus* produces egg yolk precipitation, while the above organisms react to clean the egg yolk.

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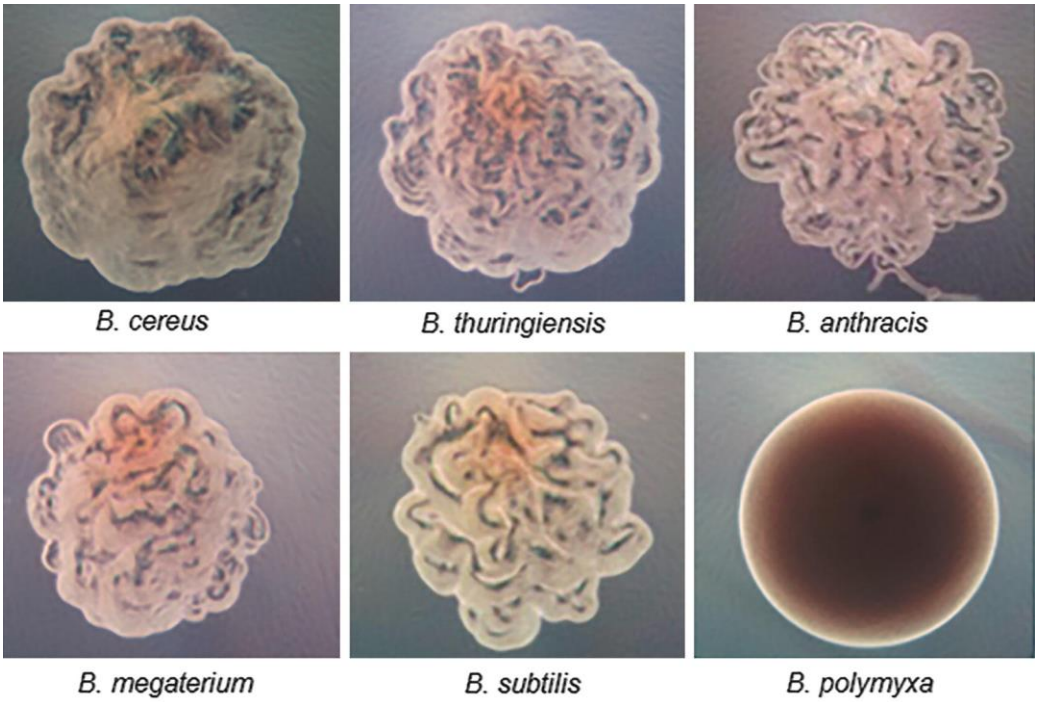

https://www.mdpi.com/2079-6374/10/3/1

Microscopic examination of fat bubbles in vegetative cells is a sign of confirmation of *B. Cereus* without biochemical analysis. Holbrook and Anderson discovered that only *B. Cereus* could form fat bubbles in vegetative cells when cultured in a selective medium. Another characteristic of the *B. Cereus* strain found in this test is that it has little or no reaction to egg yolk.

Spores of *Bacillus cereus*

a) *B. Cereus* spores are formed by endospore-formation. Under the influence of the external environment, vegetative cells differentiate and form spore morphogenesis. Under favorable conditions, mature spores, which are the beginnings of vegetative cells, are released and grow.

b) Schematic diagram of bacterial spores

Concentration of the substance in 900 ml of deionized water

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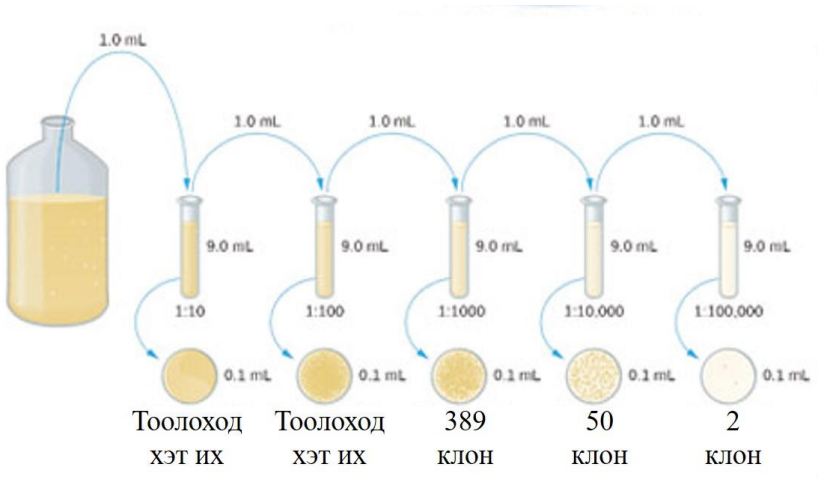
Mannitol	10.0gm
Sodium Pyruvate	10.0gm
Disodium Phosphate	2.5gm
Sodium Chloride	2.0gm
Peptic Digest of Animal Tissue	1.0gm
Dipotassium Phosphate	0.25gm
Bromothymol Blue	0.12gm
Magnesium Sulfate	0.1gm
Sterile Egg Yolk Suspension	100ml
Polymyxin B	100,000IU
Agar	15.0gm

The final pH is 7.2 +/- 0.2 at 25ºC.

Methodology for sample collection and culture:
1. Depending on the source material, it can be prepared as follows: liquid, semi-solid, solid or frozen.
2. Dilute 10 times with sterile diluent to 30-300 CFU.
3. Take 0.1 ml of the well-mixed sample and distribute it evenly on a solid medium withsterile spatula



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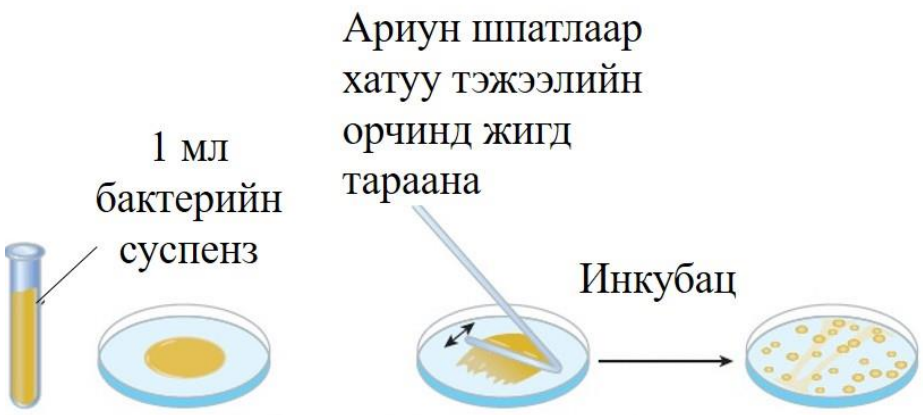

The colony of *B. Cereus* is wavy, 5 mm in diameter, blue in color, and the surrounding sediment is the same color. This distinguishes *B. Cereus* from other types of bacilli other than *B. Thuringiensis*, *Staphylococcus aureus*, *Serratia marcescens*, and *Proteus vulgaris*, which grow on egg yolks, differ from *B. Cereus* in colony morphology and color. *B. Cereus* produces egg yolk, while the above organisms undergo a reaction to clean the egg yolk.
Egg yolk reaction
In this nutrient medium, *B. Cereus* is found to be *B. thuringiensis*. Not different *from B. thuringiensis*. Then we can use the molecular method or the method of staining crystals of protein toxins. Some rare strains of *B. Cereus* respond very poorly to egg yolks and are usually unresponsive.

Quality control

Test Organisms	**Inoculation Method***	**Incubation**			**Results**
		Time	**Temperature**	**Atmosphere**	
Bacillus cereus ATCC® 13061**	A	24-48hr	35°C	Aerobic	Growth; blue colonies with opaque halo
Bacillus subtilis ATCC® 6633**	A	24-48hr	35°C	Aerobic	Growth; white colonies without halo

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Pseudomonas aeruginosa ATCC® 27853**	B	24-48hr	35°C	Aerobic	Inhibited

Publication

1. Can. 1980. *J. Microbiol.*; 26:753-7590.
2. APHA Technical Committee on Microbiological Methods for Foods. *Compendium of Methods for the Microbiological Examination of Foods,* APHA, Washington, D.C.
3. Association of Official Analytical Chemists. *Official Methods of Analysis*, AOAC, Washington, D.C.
4. American Public Health Association. *Standard Methods for the Examination of Dairy Products,* APHA, Washington, D.C.
5. U.S. Food and Drug Administration. *Bacteriological Analytical Manual.* AOAC, Arlington,
VA. www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm

IFU-10057[A]

Identify bacterial species

Recent bacteriological taxonomic studies have amplified the bacterial 16S rRNA gene to determine the nucleotide sequence of that gene and compare it with other sequences in the database. This method identifies and confirms bacterial species.

Degenerate primer

Primers	Sequences (5´-3´)	Target
27F	GAGTTTGATCMTGGCTCAG	Universal bacteria [32]
518R	WTTACCGCGGCTGCTGG	
27F	AGAGTTTGATCMTGGCTCAG	Universal bacteria [32]
1492R	TACGGYTACCTTGTTACGACTT	
ITS4_F	TCCGTAGGTGAACCTGCGG	Universal fungus [33]
ITS4_R	TCCTCCGCTTATTGATATGC	
tuf_F	GCCAGTTGAGGACGTATTCT	*Staphylococcus* [13]
tuf_R	CCATTTCAGTACCTTCTGGTAA	
Hemolysin_F	CGACCTGATTGCATTCGCCAC	*Klebsiella* modified from [14]
Hemolysin_R	TGGTCAACCCAACGATCCTG	
rpoB_F	CAGGTCGTCACGGTAACAAG	*Enterobacter* [12]
rpoB_F	GTGGTTCAGTTTCAGCATGTAC	

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IUPAC nucleotide code

- R: A or G
- Y: C or T
- S: G or C
- W: A or T
- K: G or T
- M: A or C
- B: C or G or T
- D: A or G or T
- H: A or C or T
- V: A or C or G
- N: any base
- . or -: gap

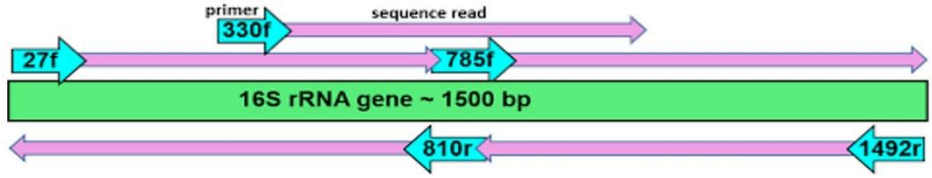
Table 1 - Primers used for the amplification of the16S rRNA.

Organism	Gene	Primer	Sequence	References
Bacteria	16S rRNA	27F	5' AGAGTTTGATCMTGGCTCAG	Suzuki and Giovannoni (1996)
		1492R	5' GGTTACCTTGTTACGACTT	
Archaea	16S rRNA	21F	5' TTCYGGTTGATCCYGCCRGA	Moyer et al. (1998)
		958R	5' YCCGGCGTTGANTCCAATT	

Underlined letters correspond to the degenerate positions: M = A + C; Y = C + T; R = A + G; N = A + G + C + T;

27F 5'-AGAGTTTGATCCTGGCTCAG-3',

1492R5'TGACTGACTGAGGY(C/T)TACCTTGTTACGACTT-3'



Identify bacterial species using PCR-amplification 16S-rRNA gene

Methodology based on research by Suzuki and Giovannoni (1996). Using the following primer, the bacterial 16SrRNA ribosome gene is amplified by PCR at 1500 bp:
Primer Name Primer Sequence 5 '3'
27F AGAGTTTGATCMTGGCTCAG
1492R GGTTACCTTGTTACGACTT

"Discrimination of Psychrotrophic and Mesophilic Strains of the Bacillus cereus Group by PCR Targeting of Major Cold Shock Protein Genes" Francis K.P., Von Stetten F., Stewart G.S., and Siegfried S, (1998). Applied and Environmental Microbiology. 64. (9), 1178-1183.
DNA sequencing procedure:
- Transfer 5 ml of bacterial culture to a 15 ml tube with 300 µl Chelex balls (prepared in 25% sterile miliQ water).
- Vortexed and centrifuged at 7000 rpm for 7 minutes
- Pour the liquid on top (supernatant).
- Dissolve 200 µl of precipitate in distilled water
- Boil for 100 ℃ 10 min to lyse the cells.
- Centrifuge at 4 ℃ for 7 min 7000 rpm
- Gently mix the supernatant (100 µl is sufficient and keep on ice). Dilute 1 aliquot 10 times in 50 µl of distilled water. The final volume is 50 µl. DNA concentrations can be measured on a spectrophotometer or NanoDrop.
Check the DNA concentration with a spectrophotometer

- DNA concentration and contamination are measured with a UV spectrophotometer at 260 nm.
- Add 98 µl of distilled water to the Epindorph tube. Add 2 µl of the isolated DNA suspension. Mix well and transfer to the cuvette of the spectrophotometer and measure with a spectrophotometer.

16SrRNA sequence for PCR replication:

PlaceE tube on ice and prepare a PCR master mix.

Reagent	25 µl
Buffer x5	5.00
MgCl2 (25mM)	1.50
dNTPs (10mM)	0.50
Primer 27F 10 µl	1.00
Primer 1492R 10 µl	1.00
ddH_2O	13.00
Taq polymerase (50ng)	0.50

- Vortex master mix
- Short time centrifuge
- Add 20 µl intoPCR tube (on ice)

Add a DNA sample
- Add 5 µl DNA samples to the first few PCR tubes
- add 5 µl positive control to the tube
- negative control of the tube, distilled water
- centrifuge the tubes for a short time

Things to keep in mind when performing work:

All operations are performed on ice.

PCR

Cycle	Температур	Хугацаа

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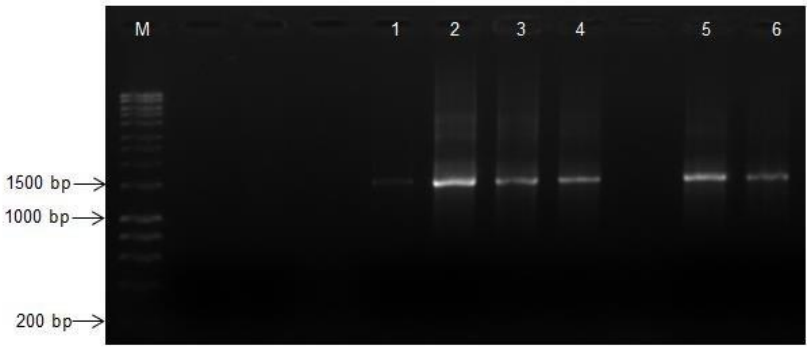
1 Cycle	95 ℃	5 мин
30 Cycles	94 ℃	30 сек
	58 ℃	30 сек
	72 ℃	40 сек
1 cycle	72 ℃	10 мин

Gel electrophoresis
Run the PCR product with 2% agarose gel containing EtB in the TBE buffer at 80V for 1.5 h.
Preparation of agarose gel
- Prepare 300 ml of 1X TBE
- Take 40 ml of 1X TBE and prepare a 2% (w / v) agarose gel. Melt, cool to 55 ℃ and add to EtBr.
- Put the comb in the gel container and freeze the gel with EtBr
- Place the gel in the electrophoresis chamber, pour in the remaining 260 ml of buffer and then remove the comb.
Gel scroll
- Add 2 µl of 6X gel loading dye to 10 µl PCR product and mix
- Pour the mixture into the gel well: the first well has a 5 µl marker, the following wells have a PCR sample, the next well has a positive control and the last well has a negative control.
- Run the TBE buffer at 80V for 1.5 hour
Results of gel electrophoresis



The figure shows a PCR product with 1500bp.

Cleaning the final PCR product.
The PCR product gel cleaning reagent shall be used in accordance with the manufacturer's instructions. When using a gel extraction kit, use low UV wavelengths to prevent primidine dimers.

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Determining the sequence of nucleic acids

Reagents used:

 ABI, BigDye Terminator v3.1 cycle sequencing kit

Sequence of analysis:

Preparation of PCR mixture

PCR mix	**Volume**
ddH_2O	4.15µl
Master mix (2.5x)	0.5µl
5x sequence buffer	1.75µl
Primer	1.6µl
Total volume	**8.0µl**

1) Add 2 µl of PCR product to each vial of PCR mixture.

2) PCR conditions:

96°C 1 min

96°C 10 sec
50°C 5 sec
60°C 4 min
} 25 cycles

4°C ∞

Cleaning of dyes from PCR products:

QIAGEN's “DyeEx 2.0 spin kit” reagent is used to remove dyes from PCR products according to the manufacturer's instructions [QIAGEN, 2011].

Determination of nucleic acid sequences:

The 3130xl Genetic Analyzer from Applied Biosystems can be used to determine the nucleic acid sequence.

Sequence alignment:

For species identification and phylogenetic analysis of bacterial strains, use the Do Blast search system of Molecular Evolutionary Genetics Analysis (MEGA) to select bacterial strains and reference strains found in other countries most similar to our strains (ncbi) placed in GenBank.

Reagents	Total volume 25 µl
Reaction buffer (5x)	5.00
MgCl2 (25mM)	1.50

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dNTPs (10mM)	0.50
Forward primer (10µM)	1.00
Reverse primer (10µM)	1.00
MQ. water	13.00
Taq polymerase (5U/µl)	0.50

Cycles	Temperature (°C)	Time
1 Cycle	95 °C	5 min
30 Cycles	94 °C	30 sec
	58 °C	30 sec
	72 °C	40 sec
1 Cycle	72 °C	10 min

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BACILLUS CEREUS SELECTIVE AGAR

Cat. no. G156	Bacillus cereus Selective Agar, 15x100mm plate, 18ml	10 plates/bag

INTENDED USE

Hardy Diagnostics Bacillus cereus Selective Agar is a selective medium for the isolation and differentiation of *Bacillus cereus* in foods.

This product is not intended to be used for the diagnosis of human disease.

SUMMARY

Holbrook and Anderson described a highly selective and diagnostic medium (PEMBA) for the isolation and determination of *Bacillus cereus* from food.[1] The medium is formulated to detect small numbers of *B. cereus* in the presence of a large number of other contaminants. Bacillus cereus Selective Agar has the ability to differentiate *B. cereus* from other species of *Bacillus* based on its resistance to polymyxin, lack of mannitol fermentation, and the presence of lecithinase. *B. cereus* can be present in a variety of produce and processed foods and the organism can cause gastointestinal illness if allowed to proliferate. Past outbreaks have been associated with the ingestion of rice, cooked meats, and cooked produce contaminated with *B. cereus.*

Hardy Diagnostics Bacillus cereus Selective Agar contains nitrogen, carbon, and meat peptone, which provide essential nutrients. Sodium chloride maintains osmotic equilibrium. The addition of sodium pyruvate enhances sporulation and egg yolk precipitation. Magnesium sulfate provides divalent cations which act as special growth factors for *Bacillus* spp. Mannitol fermentation is detected by the pH indicator bromothymol blue, resulting in *Bacillus cereus* colonies having a distinctive turquoise blue color. Egg yolk suspension is added as a source of lecithin to demonstrate the lecithinase reaction. The addition of polymyxin B inhibits gram-negative bacteria.

Typical colonies of *B. cereus* are crenated, about 5mm in diameter, and have a distinctive turquoise to peacock blue color surrounded by a zone of precipitate of the same color. These features distinguish *B. cereus* from other *Bacillus* species, except *B. thuringiensis*. Other egg yolk-reacting organisms which can grow on this medium, including *Staphylococcus aureus*, *Serratia marcescens* and *Proteus vulgaris*, are distinguished from *B. cereus* by colony morphology and color. These organisms produce an egg yolk clearing reaction in contrast to the egg yolk precipitate produced by *B. cereus.*

Microscopic examination for the presence of lipid globules in the vegetative cells is recommended as a rapid and confirmatory test for *B. cereus* and replaces the need for biochemical testing. Holbrook and Anderson have confirmed that only *B. cereus* in this

genus is capable of possessing lipid globules in its vegetative cells when grown on the selective medium.[1] One further advantage of this test is that strains of *B. cereus* that react only weakly or not at all with egg yolk can be detected and confirmed.

FORMULA

Ingredients per 900ml of deionized water:*

Mannitol	10.0gm
Sodium Pyruvate	10.0gm
Disodium Phosphate	2.5gm
Sodium Chloride	2.0gm
Peptic Digest of Animal Tissue	1.0gm
Dipotassium Phosphate	0.25gm
Bromothymol Blue	0.12gm
Magnesium Sulfate	0.1gm
Sterile Egg Yolk Suspension	100ml
Polymyxin B	100,000IU
Agar	15.0gm

Final pH 7.2 +/- 0.2 at 25ºC.

* Adjusted and/or supplemented as required to meet performance criteria.

STORAGE AND SHELF LIFE

Storage: Upon receipt store at 2-8ºC away from direct light. Media should not be used if there are any signs of deterioration (shrinking, cracking, or discoloration), contamination, or if the expiration date has passed. Product is light and temperature sensitive; protect from light, excessive heat, moisture, and freezing.

The expiration dating on the product label applies to the product in its intact packaging when stored as directed. The product may be used and tested up to the expiration date on the product label and incubated for the recommended quality control incubation times.

Refer to the document "Storage" for more information.

PRECAUTIONS

This product may contain components of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not guarantee the absence of transmissible pathogenic agents. Therefore, it is recommended that these products be treated as potentially infectious, and handle observing the usual universal blood precautions. Do not ingest, inhale, or allow to come into contact with skin.

This product is for laboratory use only. It is to be used only by adequately trained and qualified laboratory personnel. Observe approved biohazard precautions and aseptic techniques. All laboratory specimens should be considered infectious and handled according to "standard precautions." The "Guidelines for Isolation Precautions" is available from the Centers for Disease Control and Prevention at www.cdc.gov/ncidod/dhqp/gl_isolation.html.

For additional information regarding specific precautions for the prevention of the transmission of all infectious agents from laboratory instruments and materials, and for recommendations for the management of exposure to infectious disease, refer to CLSI document M-29: *Protection of Laboratory Workers from Occupationally Acquired Infections: Approved Guideline.*

Sterilize all biohazard waste before disposal.

Refer to the document "Precautions When Using Media" for more information.

Refer to the document SDS Search instructions on the Hardy Diagnostics' website for more information.

PROCEDURE

Sample collection: Consult listed references for appropriate sampling plans.[2-5]
1.Samples should be processed in a manner suitable for the source material: liquid, solid, semi-solid, or frozen.
2. Prepare appropriate decimal dilutions in sterile diluent to obtain 30-300 CFU per plate.
3. Aseptically inoculate the agar surface with 0.1ml of a well mixed diluted sample.
4. Using a sterile spreader device, spread the inoculum evenly over the surface of the agar to achieve isolated colonies.

INTERPRETATION OF RESULTS

Typical colonies of *B. cereus* are crenated, about 5mm in diameter and have a distinctive turquoise to peacock blue color surrounded by a zone of precipitate of the same color. These features distinguish *B. cereus* from other *Bacillus* species, except *B. thuringiensis*. Other egg yolk-reacting organisms which can grow on the medium, including *Staphylococcus aureus*, *Serratia marcescens* and *Proteus vulgaris*, are distinguished from *B. cereus* by colony morphology and color.

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LIMITATIONS

It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification.
On this medium, *B. cereus* is indistinguishable from *B. thuringiensis*. *B. thuringiensis* can be distinguished from *B. cereus* using molecular methods or the protein toxin crystal stain.
Occasional strains of *B. cereus* shows weak or negative egg yolk reactions.

Refer to the document "Limitations of Procedures and Warranty" for more information.

MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiological supplies and equipment such as loops, swabs, applicator sticks, other culture media, incinerators, and incubators, etc., as well as serological and biochemical reagents, are not provided.

QUALITY CONTROL

Hardy Diagnostics tests each lot of commercially manufactured media using appropriate quality control microorganisms and quality specifications as outlined on the Certificates of Analysis (CofA). The following organisms are routinely used for testing at Hardy Diagnostics:

Test Organisms	Inoculation Method*	Incubation			Results
		Time	Temperature	Atmosphere	
Bacillus cereus ATCC® 13061**	A	24-48hr	35°C	Aerobic	Growth; blue colonies with opaque halo
Bacillus subtilis ATCC® 6633**	A	24-48hr	35°C	Aerobic	Growth; white colonies without halo
Pseudomonas aeruginosa ATCC® 27853**	B	24-48hr	35°C	Aerobic	Inhibited

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* Refer to the document "Inoculation Procedures for Media QC" for more information.

USER QUALITY CONTROL

End users of commercially prepared culture media should perform QC testing in accordance with applicable government regulatory agencies, and in compliance with accreditation requirements. Hardy Diagnostics recommends end users check for signs of contamination and deterioration and, if dictated by laboratory quality control procedures or regulation, perform quality control testing to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction, if applicable. Hardy Diagnostics quality control testing is documented on the certificates of analysis (CofA) available from Hardy Diagnostics Certificates of Analysis website. In addition, refer to the following document "Finished Product Quality Control Procedures," for more information on QC or see reference(s) for more specific information.

** Recommended QC strains for User Quality Control.

PHYSICAL APPEARANCE

Bacillus cereus Selective Agar should appear translucent, and yellow in color.

REFERENCES

1. Can. 1980. *J. Microbiol.*; 26:753-7590.
2. APHA Technical Committee on Microbiological Methods for Foods. *Compendium of Methods for the Microbiological Examination of Foods*, APHA, Washington, D.C.
3. Association of Official Analytical Chemists. *Official Methods of Analysis*, AOAC, Washington, D.C.
4. American Public Health Association. *Standard Methods for the Examination of Dairy Products*, APHA, Washington, D.C.
5. U.S. Food and Drug Administration. *Bacteriological Analytical Manual.* AOAC, Arlington, VA. www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm

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