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Amplification of *Chlamydia trachomatis* in Animal Cell Host

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동물세포내에서의 유체성 세균의 증식

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Abundant amount of *Chlamydia trachomatis* could be amplified in mammalian McCoy cells and purified using descontinuous Urografin gradient centrifugation. As a chemical means to increase the *Chlamydia trachomatis* inclusions in McCoy cells IUdR treatment was found to be more effective than the cycloheximide treatment and was recommendanble for the proliferation of *Chlamydia trachomatis*. Centrifugation promoted *Chlamydia trachomatis* adhesion to McCoy cell surface, and maximal percentage of infected cells was obtained at about 3000g. The purified *Chlamydia trachomatis* could be kept in SPG solution for 48 hours at +4°C but for longer storage freezing to −70°C was necessary.

Chlamydia trachomatis is the name for a group of pathogenic bacteria causing various kinds of diseases in human being and their importance increased substantially in recent years. *Chlamydia trachomatis* strains include 15 serotypes of human origin. All members of the genus share a common antigen of polysaccharide nature and there are also many proteineous surface antigens with species- and type-specificity[1-10]. Each of these antigens possesses advantages and disadvantages in its clinical diagnosis and curing depending upon their specificity and generality.

Among the devastating diseases caused by *Chlamydia trachomatis* are trachoma, conjunctivitis, respiratory illness, inflammation in genital tract and other organs of human body. It is also the major causal agent for the human blindness and the inability to be pregnant.

These diseases usually spread implicitly making its diagnosis and treatment quite ineffective and hence throughout the world more than 400 million people are afflicted by these diseases. Although various measures have been taken in order to eliminate these diseases not a single method has been effective and the difficulty of prevention, early diagnosis and treatment is becoming more serious.

The conventional diagnosis of the infection by these pathogenic microorganisms is dependent on the cell culture and the antiserum obtained from the immunized animals but these methods have low sensitivity and specificity, and is also difficult to perform. Recently the development of diagnosis kit utilizing the monoclonal antibody from hybridoma technique caught the attention of many people as an excellent method to circumvent all the problems

Key words: ***Chlamydia trachomatis***, inclusion body, McCoy cell

associated with the conventional methods of diagnosis.

As a preliminary step toward using the monoclonal antibody against the surface antigen of *Chlamydia trachomatis* produced by hybridoma from the PEG-mediated cell-fusion in order to develop a diagnosis kit for detecting *Chlamydia trachomatis* with high precision, specificity and speed, a study was carried out to investigate various factors affecting the amplification, isolation, and preservation of *Chlamyia trachomatis* in sufficient quantity and purity.

Materials and Methods

Cell Lines and *Chlamydia trachomatis*

McCoy cells were kindly provided by Dr. H.I. Cho of the Department of Clinical Pathology, Seoul National University. *Chlamydia trachomatis* G/UW-57/Cx, D/UW-3/Cx, F/IC-Cal-3 were obtained from Dr. M.S. Lee, Department of Urology, Yon Sei University and LGV-II/434/Bubo was purchased from American Type Culture Collection, Maryland, U.S.A. P3UI, mouse myeloma cells, were kindly provided by Dr. M. Taniguchi of Department of Immunology, School of Medicine, Chiba University, Japan.

Mice

Six to 8-week-old male and female BALB/c mice were obtained from a pathogen-free colony at Experimental Animal Farms of Seoul National University.

Reagents for Cell Culture and Cell Fusion

The source of materials used in cell culture and cell fusion are listed below. MEM, RPMI fetal calf serum, phosphate buffered saline, glutamine, kanamycin solution (100x), HEPES solution, sodium bicarbonate solution (7.5%), trypsin-EDTA solution (50x), trypan blue solution, sodium pyruvate, HAT solution (50x), and HT solution (50x) were purchased from GIBCO Laboratories (Grand Island, New York). Gentamycin, vancomycin, cycloheximide, o-phenylenediamine, IUdR (5-iodo-2-deoxyuridine), guanine, L-glutamic acid, sodium phosphate, Tween 20, bovine serum albumin, peroxidase conjugated rabbit anti-mouse Igs, fluorescein isothiocyanate conjugated rabbit anti-mouse Igs, and sodium azide were purchase from Sigma Chemical Co. (St. Louis, U.S.A.). Polyethylene glycol (Mwt: 1450) from BDH, U.K. and Urografin solution from Shering, Germany, were also used.

Others

Disposable tissue culture flasks ($25cm^2$, $75cm^2$), petri-dishes (60mm), 24-well culture plates, 96-well microtiter plate, and cell scraper were purchased from Corning and Costar (Cambridge, MA, U.S.A.) and 1ml ampule for cell freezing was purchased from Nunc (Denmark). Glass conical tube (15ml, 50ml) and beaker with mesh were purchased from Abe Glass Co. (Japan). Homogenizer was purchased from Iwaki Glass Co. (Japan). Shell vials with cover slip were obtained from School of Medecine, Seoul National University. The bottles for tissue culture and all other tissue culture-related vessel were washed with 7X from Flow Laboratories, Inc. (Rockvile, Maryland, U.S.A.), rinsed thoroughly with tap and distilled water, and sterilized by dry heat (180°C, 2hours) or autoclaving (120°C, 15 psi, 20min).

Cultivation of *Chlamydia trachomatis* in McCoy Cells

McCoy cells were cultured in shell vial, tissue culture flask ($25Cm^2$) and 24 well plate for the *Chlamydia trachomatic* cultivation. In order to favor the intracellular parasitism of *Chlamydia trachomatis* by inhibiting protein and nucleic acid synthesis in the host cell[11-14] McCoy cells were treated with IUdR and cycloheximide as follows.

IUdR Treatment

McCoy cells were cultured in glass flask ($75Cm^2$). When the cells became confluent, the medium was decanted and enough prewarmed trypsin-EDTA solution was added to cover the sheet. The composition of this solution is listed in several books. After several minutes of incubating the flask at 37°C, the cell sheet appeared to have loosened from the glass

Table 1. Formula of Media for McCoy cell culture and *Chlamydia* isolation with IUdR treatment.

Ingredient	McCoy cell Growth Media.	*Chlamydia* Growth Media.
Eagles MEM	9.5g	9.5g
Fetal calf serum	100m*l*	100m*l*
Gentamycin	10mg	10mg
Glutamine	0.3g	0.3g
Sodium bicarbonate	2 g	2 g
Vancomycin	–	100 mg
Glucose	–	5 g
IUdR	12.5mg	
Total volume with distilled deionized water	1 *l*	1 *l*

*Sterilized by 0.22 μm membrane filtration

Table 2. Formula of media for *Chlamydia* storage.

Ingredient	Amount
L-glutamic acid	0.735g
Sucrose	85.575g
Sodium phosphate (dibasic)	3.581g
Total volume with distilled deionized water	1 g

*Sterilized by 0.22 μm membrane filtration and stored in aliquots at −20℃.

surface. At this time, an equal amount of MEM containing 10% (v/v) FCS was added to flask to inhibit further tryptic action, and the crude suspension was gently triturated to break up the large clumps of cells. The resulting suspension of single cells and small aggregates of cells were then collected and dispensed into sterilized cap tubes, and centrifuged at approximately 220g for ten minutes at room temperature. After centrifugation, the cells were resuspended in the McCoy cell growth media which contain the IUdR at a concentration of 12.5 mg/l (Table 1). At this time, the concentration of McCoy cells should be 10^4 cells/ml. Two-milliliter volumes of the cells suspension were transferred to flat-bottomed shell vial containing a cover slip (13mm in diameter) and each well of 24 well plate. In the case of tissue culture flask (25 cm^2), five-milliter volumes of the cell suspension were transferred. The cells were incubated in 5% CO_2 incubator at 37°C to obtain a confluent cell layer. When the cells were confluent, the medium was decanted and washed with phosphate buffered saline (PH 7.3). Three drops of the *Chlamydia trachomatis* suspension containing SPG (Table 2) were added. The inoculum was centrifuged onto the McCoy cells at 2,500xg at room temperature for 60 minutes (KUBOTA KN-70).

After centrifugation, the McCoy cells were incubated for 2 hours in 5% CO_2 incubator at 37°C, and thereafter two milliliters of *Chlamydia trachomatis* growth media (Table 1) were added. The infected McCoy cells were incubated at 37°C for 3 days, and the cells were often stained with iodine solution. The number of inclusion-forming units (IFU) was determined by examining the cells under an inverted microscope (OLYMPUS). In case of harvesting *Chlamydia trachomatis*, the iodine staining test was not carried out.

Cycloheximide Treatment

Trypsinized McCoy cells were resuspended in McCoy cell growth medium which is different from the growth medium used in IUdR treatment. (Table 3). Two-milliliter volumes of the cell suspension of which the concentration of McCoy cells are 10^4 cells/ml were transferred to shell vial and each well of 24 well plate as described above. The cells were incubated in 5% CO_2 incubator at 37°C to obtain a confluent cell layer. When the cells became confluent, the medium was decanted and cells were washed with PBS (PH 7.3). Three drops of the *Chlamydia trachomatis* suspension containing SPG were added. The inoculum was centrifuged onto the McCoy cells at 2,500xg at room temperature for 60 minutes. After centrifugation, the McCoy cells were incubated for 2 hours in 5% CO_2 incubator and two milliliters of *Chlamydia trachomatis* growth medium containing the cycloheximide at a concentration of 1mg/l (Table 3) was added. Cycloheximide is a glutarimide antibiotic which inhibits the deoxyribonucleic acid and protein synthesis of eucaryotic cells but does not affect procaryotic cells such as *Chlamydia trachomatis*. The cells were incubated at 37°C for 3 days, and inclusions were detected by inverted microscope with or without iodine staining.

Iodine Staining and Inclusion Counts

After 3 days of incubation the medium was removed from the culture wares, and the cells were rinsed with 0.9% (w/v) saline. The cells were fixed with 50%, 75% and 95% (v/v) ethanol in series for 15 minutes in each case at room temperature. The Johne's iodine-glycerin solution was added and the tubes were incubated for 20 minutes at room temperature. In case of shell vials the cover slips were removed

Table 3. Formula of media for McCoy cell culture and *Chlamydia* isolation with cycloheximide treatment.

Ingredient	McCoy cell growth media	*Chlamydia* growth media
Eagle's MEM	9.5g	9.5g
Fetal calf serum	100m*l*	100m*l*
Gentamycin	10mg	10mg
Glutamine	0.3g	0.3g
Sodium bicarbonate	2 g	2 g
Vancomycin	−	5 g
Cycloheximide	−	1 mg
Total volume with distilled deionized water	1 *l*	1 *l*

from the vials and mounted, monolayer downward, on microscope slides. Cells were examined by inverted microscope (OLYMPUS) at x100 magnification. Inclusion counts were carried out independently by at least two experienced observers, each examining either the whole cover slip three times or making counts of three sets of 10 fields chosen at random at x200 magnification with light microscope (KASSEL). An average count was calculated for the readings made by either method.

Harvest of *Chlamydia trachomatis*

Chlamydia trachomatis was havested from McCoy cell monolayers grown in 25 cm^2 polystyrene culture flasks and 24-well culture plates with 90% of the cells containing inclusions at 72 hours postinoculation. Medium was poured off and cells were removed with 4-mm glass beads in culture flasks (25 Cm^2) and by trypsin-EDTA treatment in 24-well culture plate. After the cells had been detached from culture ware, a small amount of PBS was added and next transferred into the homogenizer (IWAKI). The cells were ruptured by grinding for 3 minutes. The suspension was centrifuged at 500xg for 15 minutes at 4°C. The supernatants were collected and mixed with SPG solution (Table 2) and introduced into ampoules. The ampoules were immediately sealed. After sealing the ampoules were placed in a small styrofoam box, frozen at -20°C for 1 hour, and transferred to -70°C freezer for long term storage.

Separation and Purification of *Chlamydia trachomatis*

The cell suspensions were thawed by removing the ampoule rapidly from -70°C freezer and plunging it immediately into a water bath at 37°C-40°C. The suspensions were layered over 8ml of a 35% (v/v) Urografin solution (sodium diatrizoate and meglumine diatrizoate, 76% for injection/ Schering Berlin/Bergkamen, Germany) in 0.01 M HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) containing 0.15M NaCl and then centrifuged at 43,000xg for 1 hour at 4°C in an SW 27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellets were suspended in 0.01 M sodium phosphate (PH 7.2) containing 0.25 M sucrose and 5mM L-glutamic acid (SPG), pooled, and layered over discontinuous Urografin gradients (13ml of 40%, 8ml of 44%, and 5ml of 52% Urografin, vol/vol). These gradients were centrifuged at 43,000 xg for 1 hour at 4°C in an SW27 rotor. *Chlamydia trachomatis* bands, located at the 44/52% Urografin interface, were collected, diluted with 3 volumes of SPG and then centrifuged at 30,000 xg for 30 minutes. *Chlamydia trachomatis* pellets were washed in SPG to remove residual Urografin. Purified *Chlamydia trachomatis* was suspended in PBS and stored at -70°C.

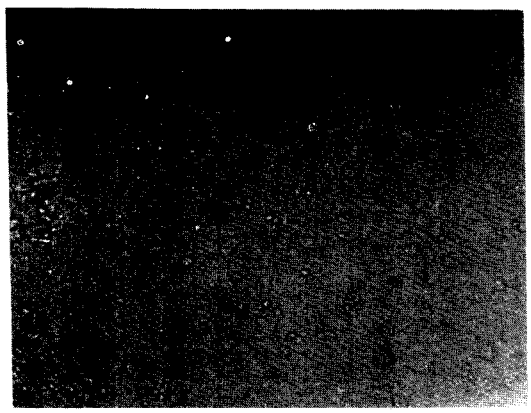

Fig. 1. Appearance of McCoy cells(100 X Magnification)

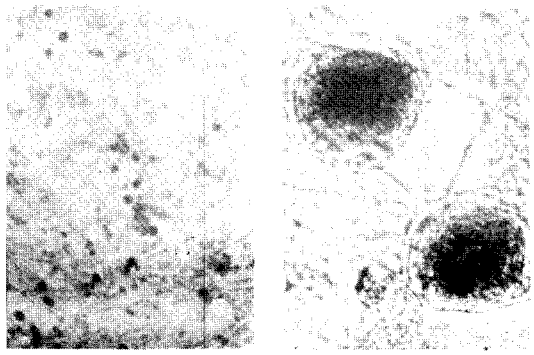

Fig. 2- a. McCoy cells with iodine-stained *Chlamydia* inclusions(100X Magnification)
b. McCoy cells with iodine-stained *Chlamydia* inclusions(1000X Magnification)

Results and Discussion

McCoy Cell Culture and Infection with *Chlamydia trachomatis*

To cultivate *Chlamydia trachomatis* within animal cells McCoy cells were cultured on coverslips, 24 well culture plate and culture flask (25 Cm^2).

Confluent cell monolayers on each culture ware were indistinguishable, all having a pavement-like appearance (Fig. 1). When the cells became confluent, McCoy cells were treated with one of two chemicals, such as IUdR and cycloheximide, and *Chlamydia trachomatis* serotypes D,F,G and L2 were inoculated. *Chlamydia trachomatis* grew and pro-

Table 4. Appearance of McCoy cells and iodine stained inclusions.

Cell Treatment	Morphology of McCoy Cells*	Size*	Inclusions Brightness**	Inclusion* forming units
Untreated	+ +	+	+ +	+
Cycloheximide	+ +	+ +	+ + +	+ + +
IUdR	+ + +	+ + +	+ +	+ +

*: Symbols refer to an arbitrary scale from smallest (+) to largest (+ + +)
**: Symbols refer to an arbitrary scale from poorest (+) to best (+ + +)

duced inclusions which were easily detectable in all types of cell monolayer. The produced inclusions were well stained with iodine staining solution (Fig. 2-a, Fig. 2-b). This is due to glycogens which are produced during the multiplication of *Chlamydia trachomatis* within McCoy cells. As shown in Figure 2-b, *Chlamydia trachomatis* organisms were aggregated in the cytoplasm of McCoy cell.

Treatment of McCoy Cells for the Proliferation of *Chlamydia trachomatis*

The exposure of McCoy cells to various chemicals enhances susceptibility to chlamydial infection by inhibiting host cell protein and nucleic acid synthesis, thereby increasing the availability of nutrients for the parasite. The most common chemicals used for the treatment of McCoy cells include 5-iodo-2'-deoxy-uridine (IUdR) and cycloheximide. Only limited information is available on the relative efficacy of chlamydial infection into the untreated McCoy cells and the cells treated with the two aforementioned chemicals.

A comparison was made among the untreated, the IUdR-treated, and the cycloheximide-treated McCoy cells as hosts for *Chlamydia trachomatis* in order to obtain the cytoplasmic inclusions of better quality and quantity and thus to maximize the antigen production. The morphology of the McCoy cells and the quality of the inclusions were best with IUdR-treated cells as compared with the other treatments (Table 4). The inclusions were large and brightly stained. The McCoy cells were also large and multinucleated. The McCoy cells and inclusions in the case of cycloheximide treatment were smaller than those from IUdR treatment, although the cycloheximide-treated cells yielded slightly smaller number of inclusions. As the optimal objective is to obtain the largest number of *Chlamydia trachomatis* organisms from McCoy cells IUdR treatment system was more efficient than cycloheximide-treatment and non-treatment system, and was recommendable for the isolation of *Chlamydia trachomatis* in McCoy cells.

For the diagnosis of *Chlamydia trachomatis*, cycloheximide-treated McCoy cells proved more efficient than McCoy cells treated with IUdR since the cycloheximide method has the advantages of time saving and higher sensitivity.

Effect of Centrifugal Force

Chlamydia trachomatis attachment is an essential prerequisite to ingestion[15,16]. It is generally known that association of *Chlamydia trachomatis* with the cell surface is relatively weak, time being necessary to stabilize *Chlamydia trachomatis* attachment through the formation of multiple binding sites. Multiple binding is facilitated by movement of the cell membrane immediately adjacent to the *Chlamydia trachomatis* particle. The slow rate of *Chlamydia trachomatis* attachment severely limits the rate of *Chlamydia trachomatis* uptake into the McCoy cell so that ingestion occurs over a wide range of time[17,18]. Centrifugation promotes *Chlamydia trachomatis* attachment mechanically and helps overcome electrostatic barriers to attachment.

In this study, centrifugal forces of 1000, 2000, 3000 and 3800xg were applied to chemically treated McCoy cells using bench-top centrifuge (KUBOTA). All centrifugations were carried out for an hour at room temperature.

The results (Fig. 3) indicate that below about 3000xg the percentage of infected cells were proportional to the centrifugal force applied. At about 3000xg, however, maximal percentage of infected cells was obtained. At 3800xg, the maximum centrifugal force that can be generated by the bench-top centrifuge available in our laboratory, no significant improvement in the adhesion could be observed. This confirms

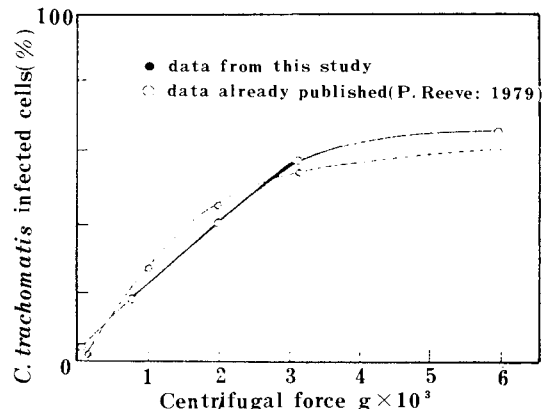

Fig. 3. Relationship between the percentage of *C. trachomatis* infected cells and the centrifugal force applied to infect cell cultures.

similar result reported by Reeve and Owen[19].

Effect of Storage

In order to assess the effects of storage on the infectivity of *Chlamydia trachomatis*, suspensions of the L2/434/Bu strain in SPG were either stored at 4°C or frozen to -70°C, and infectivity was measured by counting the number of inclusions formed after incubation in IUdR treated McCoy cell monolayers. Over 80% infectivity was lost after storage at +4°C for 48 hours (Table 5), and thereafter infectivity continued to diminish.

Although a similar drop in initial infective titre occurred with material frozen at -70°C, no further infectivity losses could be discerned. However, subsequent thawing for isolation attempts reduced the infectivity of *Chlamydia trachomatis* suspensions by about one-third.

Separation and Purification of *Chlamydia trachomatis*

McCoy cells infected with *Chlamydia trachomatis* were harvested from tissue culture flasks (25 cm^2) or 24 well plates by glass beads or trypsinization. The cells were ruptured by grinding with homogenizer and centrifuged at 500xg. The impure *Chlamydia trachomatis* suspension was further purified by discontinuous Urografin gradients. Chlamydia trachomatis cells were mostly located at the 44/52% Urografin interface (Fig. 4) and some settled down with McCoy cell debris[20,21]. The collected *Chlamydia trachomatis* organisms from 44/52% interface were washed with SPG solution several times and resuspended in PBS.

Conclusion

McCoy cells were cultured in shell vial, tissue culture flask (25 Cm^2) and 24 well plate for the cultivation of *Chlamydia trachomatis*. Large amount of *Chlamydia trachomatis* organisms could be amplified in IUdR or cycloheximide treated McCoy cells. IUdR treatment gave better results than cycloheximide treatment. The optimal centrifugal force required for obtaining maximal percentage of infected cells was about 3,000xg.

Chlamydia trachomatis in SPG solution could be kept at +4°C for 48 hours but for longer storage freezing to -70%C was necessary.

The impure *Chlamyia trachomatis* suspension was purified by discontinuous Urografin gradient centrifugation. *Chlamydia trachomatis* cells were mostly located at the 44/52% Urografin interface.

Table 5. Effect of storage at +4℃ and −70℃ of *Chlamydia trachomatis*

No. of days Stored	No. of inclusions per cover slip. Storage at +4℃	Storage at −70℃
0	Ca. 200	Ca. 180
1	80	30
2	35	40
3	10	25
4	0	35
5	0	35
6	0	20
7	0	25

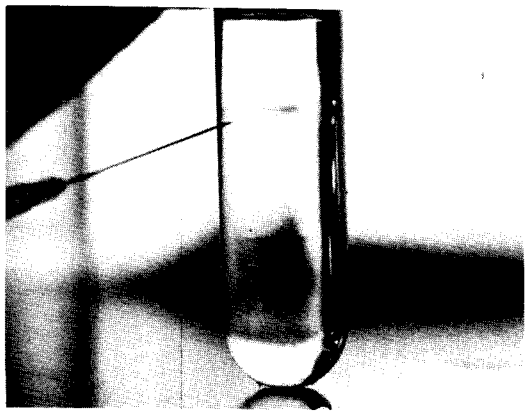

Fig. 4. The purified *C. trachomatis* EB band from discontinuous urografin gradient centrifugation

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요 약

*Chlamydia trachomatis*는 인간에게 각종 질병을 일으키는 병원균이며 최근 그 중요성이 크게 증가하였다.

Chlamydia trachomatis 감염에 대한 재래식 진단

방법은 낮은 정밀도와 낮은 특이성을 갖고 있으며 방법의 수행 자체도 어렵다는 문제점을 지니고 있다. 이들 재래식 진단방법들이 안고 있는 문제점들을 해결하기 위하여 항-병원균 모노클로날 항체를 생산하는 하이브리도마 세포주를 만들 최종목표를 갖고 우선 생쥐에 면역시킬 항원으로 사용할 ***Chlamydia trachomatis***를 동물 숙주 세포내에서 증식시키는데 끼치는 각종 인자들에 대한 영향을 조사 연구하였다.

동물 세포 유래의 McCoy 세포 내에서 충분한 양의 ***Chlamydia tracthomatis***를 증식시킨후 불연속 Urografin 구배 원심분리에 의해 정제하였다. McCoy 세포 내에서의 ***Chlamydia trachomatis***의 생성 개체수를 늘리기 위하여 화학제제를 사용했던 바 IUdR 처리가 cycloheximide 처리보다 더 효과적이었으며 ***Chlamydia trachomatis***증식을 위해 쓸 수 있는 방법이었다. 원심력의 증가가 ***Chlamydia trachomatis***의 McCoy 세포 표면에의 흡착을 증진시켰으며 약3000g 근처에서 최대 감염율을 보였다. 분리 정제한 ***Chlamydia trachomatis***는 SPG 용액 내에서 4℃에서 48시간동안 저장될 수 있었으며 더욱 오래 동안 저장시키기 위해서는 −70℃ 까지 냉동시키는 것이 필요하였다.

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