

## CHICK EMBRYONIC KIDNEY CELL ISOLATION AND CRYOPRESERVATION

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### SUMMARY

Chicken kidney cells are important sources of primary cultures for virus study as well as vaccine production. The preparation of primary culture requires expertise as the risk of contamination is often very high. Yields of primary culture preparation are an important factor, especially when cells are used in vaccine production. The ability to cryopreserve primary cells is another important factor that needs to be taken into consideration since large stock must be available for urgent situations. In this study, kidneys of eighteen day old chick embryo were isolated and cold trypsin procedure was used to prepare the primary culture. Cold trypsin tissue dispersion is suitable for embryonic tissue and it is less arduous than the common warm trypsin procedure. Cells were seeded at  $10^6$  cell/ml and maintained in BME supplemented with 5 - 10% FBS, 0,3% tryptose phosphate broth and 1% antibiotic-antimycotic at 38.5°C with HEPES as the main buffer system. Confluent monolayer was consistently obtained after 3 - 4 days. Yields for cold trypsin method were consistently 2 to 3 fold higher than those of warm trypsin method. Recovery rates after 24 h in culture were comparable to that of warm trypsin (74 - 78%). Initial results for *in situ* cryopreservation showed that it was possible to preserve chick embryonic kidney cells at  $-86^{\circ}\text{C}$  while attached to culture substrates. The cryopreservation procedure does not require slow cooling step that is usually required in standard cryopreservation procedure. Chick embryonic kidney cells, prepared by this method are also permissive to influenza virus infection, as cytopathic effects typically appear two days after inoculation.

**Keywords:** Chick embryonic kidney (CEK) cells, cold trypsin, warm trypsin, *in situ* cryopreservation, influenza virus

### INTRODUCTION

Primary chick kidney (PCK) and chick embryonic kidney (CEK) are widely used in studying poultry viral diseases (Marek, ILTV, bronchitis, influenza, Newcastle *etc.*) and vaccine production (Cook *et al.*, 1975; Davison, Nair, 2004; Wambura *et al.*, 2006; Wark *et al.*, 1979). They have also been used to study human influenza virus as well as in human vaccine production (Maassab, Bryant, 1999; Tannock *et al.*, 1985).

Tannock *et al.* (1985) reported protocols for large scale production of PCK & CEK for human influenza vaccine production. In this report he suggested cold trypsin procedure for preparation of CEK which was suitable for large scale production (Tannock *et al.*, 1985). However, there has been no report on cold trypsin procedure for CEK preparation since then. Most protocols reported in the literature for CEK preparation are warm trypsin protocol (Choi *et al.*, 2007; Tannock *et al.*, 1985; Wambura *et al.*,

2006). This protocol usually takes 3 - 4 hours to complete (Tannock *et al.*, 1985) and thus not convenient for large scale production. Therefore, in this study we attempted to prepare CEK cells by cold trypsin method, and yields for CEK by cold trypsin method would be reported for the first time.

Tannock *et al.* (1985) reported failing to cryopreserve any of PCK and CEK (Tannock *et al.*, 1985). Since vaccine production requires large amount of cultures available, thus cryopreservability of a culture is a very important criterion for its selection as the substrate. Choi *et al.* (2007) reported succeeding in cryopreserving CEK cells in liquid Nitrogen (Choi *et al.*, 2007). However, since CEK cells will not go pass 5<sup>th</sup> passage (Smith *et al.*, 2007), we feel that it is not economic to cryopreserve these cells in liquid nitrogen. Furthermore, the 20% dimethyl sulfoxide (DMSO) used in this procedure was commonly regarded as very toxic to the cells (Freshney, 2005). Here in this study we would attempt to cryopreserve CEK cells at  $-86^{\circ}\text{C}$  while

attached to the substrate, a procedure which was coiled *in situ* cryopreservation by Ohno *et al.* (1991).

## MATERIALS AND METHODS

Embryonated eggs were purchased from a reputable poultry breeding farm (Hong sanh Hatchery) at 7 to 14 day old and incubated to 18 day old at 37°C with daily turning. (Though these are not specific pathogen free eggs, which are required for human vaccine production, they have been routinely used for veterinary vaccine production by some companies in the area, such as Navetco. Therefore we feel that they are sufficiently good for our study.)

Dissecting buffer was prepared as either PBSA or DBSS supplemented with antibiotics (streptomycin, penicillin and kanamycin). Cold trypsin 0.25% was prepared by dissolving 0.25 g crude trypsin (Sigma) into 100 ml of RPMI 1640 (Sigma) and kept stirring on magnetic stirrer for 1 hour. The solution was then sterilized by filtration with 0.2 µm microfilter (Millipore) and stored at 4°C for no more than 3 weeks. Trypsin/EDTA 0.25% was prepared as 2.5% stock by dissolving 2.5 g crude trypsin and 0.372 g EDTA in 1L of PBSA and diluted 1:10 before use.

Cells were maintained in Basal Minimum Essential (BME) medium (Sigma) supplemented with 5 - 10% FBS (Gibco), 0.3% tryptose phosphate broth (w/v) (Sigma) and 1% antibiotic-antimycotic (Sigma). DMEM/F12 supplemented with 10% or 20% DMSO (Sigma) was used as cryopreserving medium. All media and solutions are kept at 4°C for no more than 3 weeks.

Cells were cultured in either 25 cm<sup>2</sup> non-vent cap or vent cap T-flask (Nalge Nunc or Corning), or 6 well dishes (Corning Costar) at 38.5°C in Sanyo CO<sub>2</sub> incubator with 5% CO<sub>2</sub> setting. Hepes was the major buffer system in the culture.

### Cold trypsin procedure for CEK preparation

Eighteen day old embryonated eggs were swabbed with 70% alcohol and immersed in 70% alcohol for 5 minutes, then transferred to an egg cup or 50 ml beaker. Egg shell was cracked with sterile

forceps at the air sac area and chorio-allantoic membrane removed. The embryo was lifted out with sterile forceps and severed from the yolk sac with sterile scissors. The embryo was transferred to a Petri disc and cut at the centre, near under the wings. Viscera were removed and kidneys were taken out with sterile forceps (note that avian kidneys have an elongate, three lobed kidney instead of bean shape kidney like mammalians). Kidneys were collected into a 50 ml beaker and washed extensively with dissecting buffer by gently pipetting up and down. New beaker should be used for each washing (at least 2 times). After the final wash, kidneys were transferred to a pre-weighed sterile glass tube with screw cap. The tubes were re-weighed to determine the weight of the kidneys (wet weight).

Ten ml 0.25% trypsin/RPMI 1640 at 4°C was added to every one gram of tissue. The mixture was placed in the refrigerator at 4°C for 6 - 24 h.

After 5.5 h/24 h in the refrigerator, the tubes were taken out and trypsin was carefully removed and discarded, leaving the tissue with only residual trypsin. Tubes were placed into the incubator at 37°C for 20 - 30 minutes. Approximately 1 ml of pre-warmed maintaining medium was added to every 100 mg of original tissue and gently pipette the mixture up and down until the kidneys are completely dispersed. Add more medium, approximately 6 - 10 ml and allow large pieces to settle (usually take 3 - 5 minutes). Carefully collect the supernatant into a 15 ml centrifuge tubes and centrifuged at 80 - 100 g for 10 minutes. Cells were resuspended into a minimal volume of medium (1 ml/100 mg tissue) and an aliquot was taken out for quantification with a hemocytometer (Improved Neubauer, Germany). After yield and viability ratio were obtained, cells were seeded into culture containers at 10<sup>6</sup> cells/ml.

Twenty four hour later the flasks were taken out of the incubators and medium was discarded. Monolayers were washed with PBSA to remove unattached or loosely attached cells. Monolayer was then trypsinized with 1 ml 0.25% trypsin/EDTA for 20 - 30 minutes and dispersed cells were counted with a hemocytometer. The 24 h recovery rate was calculated as follow:

$$\text{Recovery rate} = \left[ \frac{\text{Total No of viable cells seeded}}{\text{Total No of attached cells after 24 hour}} \right] \times 100\%$$

### Cryopreservation

CEK cells were maintained at 38.5°C for 3 - 4 days until confluent monolayer was obtained. Medium was discarded and replaced with DMEM/F12 supplemented with 10% FBS. The cultures were closely monitored for further 24 hour for signs of contamination. Seven flasks with healthy confluent monolayers and no sign of contamination were used in cryopreservation experiment. Medium was aspirated and monolayers were washed with PBSA. 2 - 4 ml of cryopreserving medium was added into each flask. Three flasks were added with DMEM/F12 supplemented with 20% DMSO and the rest were added with DMEM/F12 supplemented with 10% DMSO. Flasks were put directly into the -86°C freezer for 3 days. After 3 days flasks were taken out and thawed in a 37°C incubator for 30 minutes. After the cultures were completely thawed, medium was aspirated and cells were washed twice with pre-warmed maintaining medium (DMEM/F12 containing 10% FBS). New medium was added and the cultures were transferred to the incubator. Cell recovery was checked every 24 hour with inverted microscope.

### Viral infection

Confluent CEK cells were inoculated with NIBRG-14 influenza strain at 0.1 Multiplicity Of Infection and allowed for viral adsorption for 30 minutes. Cells were then washed with PBS and maintaining medium was added. No extraneous trypsin was added. Cells were observed for cytopathic effects at regular interval (24 hours) and pictures were taken with digital camera. Negative controls were inoculated with PBS. This experiment was carried out at HoChiMinh Pasteur Institute and Navetco laboratory.

### RESULTS AND DISCUSSION

Yields, viability and 24 h recovery rate of CEK cells prepared under cold trypsin conditions were presented in the table 1. Yields, viability and 24 h recovery rate of CEK cells prepared by warm trypsin method in other studies (Choi *et al.*, 2007; Tannock *et al.*, 1985) were included in the table for comparison. Three 18 - 19 day old embryonated eggs were used in each independent experiment. Only replicas that showed no sign of contamination were reported in this study.

Of seven flasks which were cryopreserved, all

showed good sign of recovery and 3 out of 7 flasks recovered completely as original after only 36 hours. Recovery did not appear to correlate with concentration of DMSO.

To complete the cryopreservation protocol, cells should be cryo-preserved for several months and periodically thawed and the recovery rate monitored to assess the efficiency of the procedure. Due to some objective reasons we were unable to complete this step in this study.

CEK cells have been used in several poultry virus studies (Cook *et al.*, 1975; Davison, Nair, 2004; Wambura *et al.*, 2006; Wark *et al.*, 1979). They had also been used to produce vaccines for poultry and human (Maassab and Bryant, 1999; Wark *et al.*, 1979). Therefore, a protocol for large-scale production of CEK cells would be very beneficial to vaccine industry. Tannock *et al.* (1985) suggested cold trypsin procedure for large scale CEK culture preparation, but they did not report any protocol nor any data (Tannock *et al.*, 1985). Our results showed that cold trypsin method was not only very flexible but also gave higher yields compared to warm trypsin. Kidneys can be removed continuously during the day and cell disaggregation step can be synchronized in the next morning. The yields are always twice or three times higher than those of warm trypsin while recovery rates are comparable.

Tannock *et al.* reported unable to cryopreserve CEK cells in liquid nitrogen (Tannock *et al.*, 1985). Choi *et al.* succeeded in cryopreserving CEK cells with medium containing 20% DMSO. They emphasized that the key factor for the success was the 20% DMSO (Choi *et al.*, 2007), which is traditionally used at 5 - 15%. However, our literature review showed that primary culture were best cryopreserved at -86°C while attached to substrate. Though *in situ* cryopreservation is only good for short term preservation (6 months to 1 year), the method is cheap and suitable for CEK cell line, which normally do not live beyond 5<sup>th</sup> passage. Our preliminary results showed that the success for cryopreservation of CEK cell might not lie in the concentration of DMSO (as asserted by Choi *et al.*) but rather the method for cryopreservation. This speculation agrees with results from Ohno *et al.* (1991). Furthermore, they also demonstrated that the slow cooling step in most standard cryopreservation procedures can be eliminated without affecting the success of the procedures.

Bird flu recently has inflicted great economic loss and high dead toll on populations of several countries. Experts agreed that the only effective measure for containment is vaccination (Horimoto, Kawaoka, 2006). Influenza vaccines were traditionally produced in SPF embryonated eggs which may be subjected to shortage in case of epidemics. Furthermore, in egg-based vaccine production there is always a 4 to 6 month lead time for organization of egg supply. Cell culture based vaccine production offers several advantages over egg-based vaccine production. Cell lines used in cell culture-based vaccine should satisfy certain criteria such as being able to propagate the virus to high titre and permissive to a wide range of influenza strains (Rappuoli, 2006). CEK cells might be a suitable candidate for cell-based vaccine production. Firstly, they are able to propagate several strains of influenza virus, including those that have not been previously adapted to CEK cells (Smith *et al.*, 2007). Secondly, they are able to maintain the identity of HA and NA

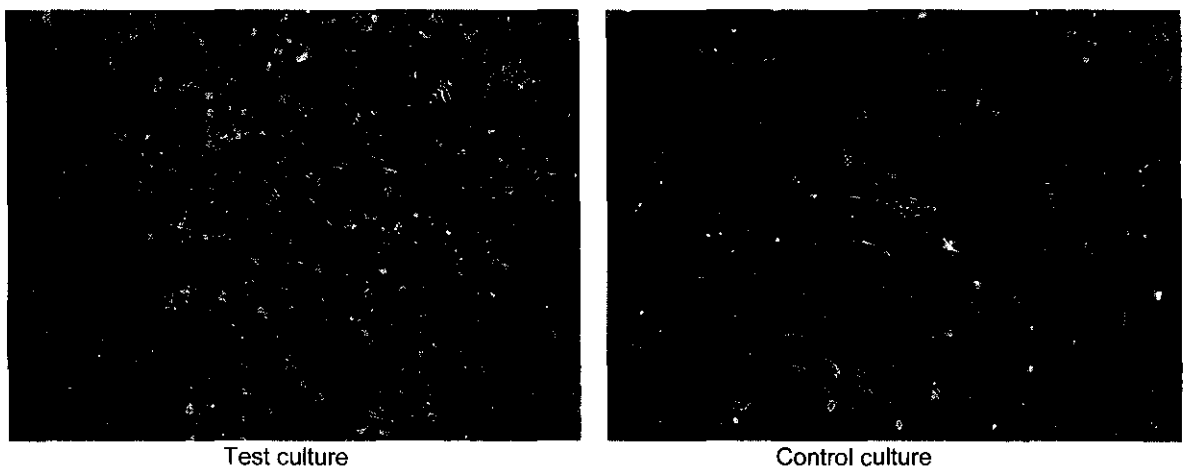
of the newly formed virions faithfully to the original viruses, a factor that is crucial to the effectiveness of an influenza vaccine (Katz, Webster, 1992). Thirdly, CEK cells can propagate influenza virus without addition of trypsin (Kaverin, Webster, 1995). Fourthly, CEK cell can be prepared by cold trypsin method, which gives higher yield than warm trypsin method (adult tissue can not be used with cold trypsin procedure). Finally, CEK cells can be cryopreserved, either in liquid nitrogen or in  $-86^{\circ}\text{C}$  freezer.

To test our CEK cells as a substrate for influenza virus replication we inoculated several cultures with NIBRG-14 strain and monitor for cytopathic effects. Due to hazardous nature of the procedure, the Navetco company and Hochiminh Pasteur institute staff had kindly carried out the experiment for us. Cytopathic effects, appear as cell detachment from the culture vessels' bottom, typically appear 48 hours after adsorption (Figure 1).

**Table 1.** Yields, viability and 24h recovery rate of CEK cells prepared under cold trypsin conditions & in comparison with warm trypsin procedure.

Experiments	Yield per pair of kidney (cells)	Viability determined by dye exclusion (%)	Recovery rate after 24h culture (%)
Incubated at $4^{\circ}\text{C}$ for 5.5 h <sup>1</sup>	$4.3 \cdot 10^7 \pm 0,25$	$73.63 \pm 2,8$	$74 \pm 0,1$
Incubated at $4^{\circ}\text{C}$ for 24 h <sup>2</sup>	$3 \cdot 10^7 \pm 0,6$	$68.33 \pm 4,9$	$78 \pm 0,2$
Warm trypsin <sup>3</sup>	$1.21 \cdot 10^7$	80%	62.25 - 81.25

<sup>1</sup>Values represent 6 independent experiments; <sup>2</sup>Values represent 3 independent experiments; <sup>3</sup> The results are obtained from other studies, in which statistical data were not available. Values are mean  $\pm$  SD



**Figure 1.** CEK cells 48 hour after viral adsorption (x20).

## CONCLUSION

Cold trypsin method is a more convenient and effective method of CEK cell preparation than warm trypsin method. Yields are always twice or three times higher than those of warm trypsin procedure. Initial study for *in situ* cryopreservation of CEK cells showed promising results. Cytopathic effects can be observed in CEK culture prepared by cold trypsin two day after influenza virus inoculation.

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## THU NHẬN VÀ BẢO QUẢN TẾ BÀO THẬN PHÔI GÀ

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### TÓM TẮT

Tế bào thận gà là một nguồn tế bào sơ cấp có giá trị cho việc nghiên cứu và virus và sản xuất vaccine. Quá trình thu tế bào sơ cấp nhìn chung khá phức tạp bởi khả năng nhiễm rất cao. Hiệu suất thu

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tế bào sơ cấp là một yếu tố quan trọng, đặc biệt trong trường hợp tế bào được sử dụng để sản xuất vaccine. Khả năng bảo quản được tế bào sơ cấp là một yếu tố quan trọng khác cần được xem xét bởi phải luôn dự trữ một lượng đủ để đối phó với trường hợp khẩn cấp. Phương pháp trypsin lạnh phù hợp để tách tế bào từ mô phôi và thường không vất vả và tốn thời gian như ở phương pháp trypsin ấm. Trong nghiên cứu này phôi gà 18 ngày tuổi được thu theo phương pháp trypsin lạnh và nuôi trong môi trường BME bổ sung 10% FBS, 0,3% tryptose phosphate broth (w/v) và 1% kháng sinh (v/v) ở trong đĩa 6 giếng hoặc bình cấy T-flask 25 cm<sup>2</sup>. Tế bào thường mọc kín giá thể nuôi sau 3 - 4 ngày trong điều kiện nhiệt độ nuôi 38,5°C với hệ đệm chủ yếu là HEPES. Hiệu suất thu tế bào của phương pháp trypsin lạnh luôn cao hơn 2 đến 3,5 lần so với phương pháp trypsin ấm. Khả năng phục hồi của tế bào thu bằng phương pháp trypsin lạnh sau 24 h nuôi là từ 74% đến 78%. Thử nghiệm bảo quản bước đầu cho thấy có thể bảo quản tế bào thận phôi gà bằng phương pháp *in situ*. Thử nghiệm ban đầu cho thấy tế bào thận phôi gà thu bằng phương pháp trypsin lạnh cũng nhân được virus cúm và bệnh tích thường xuất hiện sau hai ngày kể từ lúc nhiễm.

**Từ khóa:** Bảo quản *in situ*, tế bào thận phôi gà, trypsin ấm, trypsin lạnh, virus cúm