Chapter 7

The Preparation of Chicken Kidney Cell Cultures for Virus Propagation

Elena Lokhman, Srijana Rai, and William Matthews

Abstract

Chicken kidney (CK) cells have been widely utilized in virus research studies for many years. The optimized technique of primary CK cell culture production involving both mechanical and enzymatic disaggregation is described. This updated method proved to consistently give high cell yields and resultant cultures are readily used for virus assays.

Key words  Chicken kidney cells, Primary cultures, Virus propagation, Viral assays

1 Introduction

Primary chicken kidney cultures have always been widely used for research purposes and propagation of various viruses [1, 2]. Chicken embryonic kidney (CEK) cultures are known to have high susceptibility to enzymatic disaggregation and great proliferation potential. However, kidneys from a few days old embryos are hard to excise and cell yields are quite low. Young chicken kidneys contain more connective tissue and are generally more difficult to dissociate as compared to embryonic tissues. Nevertheless, they give higher cell yields and resulting cultures are susceptible to infection with different animal viruses [1, 3].

Kidney cultures from young birds are routinely mentioned in literature as a successful system for virus studies and even primary virus isolation [1, 4–6], occasionally showing higher sensitivity to viruses than CEK cells [7]. In particular, primary CK cells are an important tool in avian Gammacoronavirus infectious bronchitis virus (IBV) research. The ability of CK cells and ex vivo chicken tracheal organ cultures (TOCs) to support the growth of various IBV strains enabled the comprehensive study conducted by Maier et al. [8] on IBV induced membrane rearrangements described in previous studies [9, 10] and their potential role in the process of
virus replication. This work focused on an in-depth comparison of types, shape and size, abundance, dynamics and temporal and spatial co-occurrence of membrane rearrangements induced by a substantial set of IBV strains, including pathogenic and apathogenic field and vaccine strains.

The use of CK cells has also allowed for comparisons of the growth kinetics of naturally occurring strains against recombinant IBVs [11]. It has previously been discovered that spike (S) glycoprotein of the virus envelop is responsible for extended cellular tropism exhibited by particular IBV strains [12]. Recombinant IBV viruses were constructed and screened to expand on this finding and determine which S protein subunit is exactly imparting this feature [11]. Another study focused on RT-qPCR as a method for quick detection of early IBV infection. Authors showed that choosing the genes of appropriate transcriptional stability is one of the key factors in correct analysis of RT-qPCR data as transcriptional profiles vary in different IBV-infected cell types, which was demonstrated by using CK cells along with DF1 cell line as two model systems [13].

In this chapter, we describe an improved protocol for primary CK cells isolation, a modification of method described previously by Hennion and Hill [14]. The introduced changes doubled the yield of primary chicken kidney cells, improved quality of obtained monolayers and reduced the overall laboriousness of the method.

## 2 Materials

### 2.1 Extraction of Kidneys

1. 2–3 week old chicken(s) from specific pathogen free (SPF) flock culled by cervical dislocation and death confirmed by cessation of the heart.
2. Sterile phosphate buffered saline without calcium and magnesium (PBSa).
3. 70% ethanol and decontaminating wipes.
4. Sterile instruments to include two pointed scissors and one round ended forceps.
5. Skirted falcon tubes and rack.
6. Sterile bioassay dish.
7. Clinical waste bags and cable ties.
8. Class II microbiological safety cabinet (MBSC).

### 2.2 Isolation and Culture of Kidney Cells

1. Sterile instruments to include tweezers and two disposable scalpels.
2. Sterile Petri dish.
3. Sterile glassware to include two 1 l and one 500 ml baffled flasks, 250 ml beaker, 250 ml wide-necked conical flask and two 100 ml measuring cylinders.

4. Sterile magnetic stirrer bar of suitable size for 500 ml baffled flask.

5. Sterile 50 ml syringe.

6. Sterile glass funnel with two layers of muslin to act as a filter.

7. Sterile wire mesh (mesh No. 50 × 0.200 mm diameter wire, 0.308 mm aperture) folded into a filter shape to fit a funnel (see Note 1).

8. Sterile Swinnex 25, reusable, syringe driven polypropylene filter unit fitted with metal gauze mesh No. 100 × 0.100 mm diameter wire, 0.154 mm aperture.

9. Sterile 250 ml plastic centrifuge tubes.

10. Sterile serological pipettes and pipette gun.

11. Tissue culture grade flasks or plates.

12. Pipettes and sterile filtered tips.

13. Centrifuge.

14. Magnetic stirring platform with heating.

15. Haemocytometer.

16. Trypan blue.

17. Inverted microscope suitable for observing cell cultures.

18. Incubator set at 37 °C and 5% CO₂.

19. Sterile PBSa.

20. Newborn calf serum (NBCS).

21. Trypsin-EDTA solution: 0.5 g/l porcine trypsin and 0.2 g/l EDTA·4Na(HBSS).

22. Growth medium: Eagles Minimum Essential Medium (EMEM) supplemented with 10% NBCS, 10% Tryptose phosphate broth (TPB), 2 mM L-glutamine, 10 mM HEPES, 20 U/ml penicillin and 0.02 mg/ml streptomycin.

3 Method

The kidneys are removed immediately after confirmation of death to minimise the build-up of blood clots and maintain viability of the cells. We have consistently observed the average yield of $4 \times 10^8$ to $5 \times 10^8$ CK cells from a 2–3 week old Rhode Island Red bird, which is more than a two-fold increase compared to the previously reported yields [14].
3.1 Extraction of Kidneys

1. After culling, place the birds on a bioassay dish and spray the back and under the wings with ethanol to clean and dampen the feathers before placing inside the MBSC.

2. Insert the blade of a clean pair of scissors just below where the wing attaches to the body and sever across the body through the spinal cord. From one end of this cut, angle scissors towards the legs and cut through the rib cage stopping just before the femur (leg bone), taking care to avoid piercing any internal organs.

3. Repeat this from the opposite end of the first cut to cut down other side of bird towards the other femur (see Note 2).

4. Using your hands, place thumb on the severed part of the spine and pull until the inner organs are exposed and the carcass lays open on its own. Push the bird’s legs (by the knees) backwards until the ends of the femurs pop out, which enables the legs to stay flat.

5. Carefully fold back the cut section of the bird to reveal the internal organs. Gently move intestines aside to expose the kidneys. Use another pair of clean scissors to cut any fatty tissue or membrane to minimize resistance and the chance of tearing intestines.

6. To extract cut along both sides of each kidney, then cut along underneath whilst pulling the kidney up with forceps. Place kidneys into falcon tube containing PBSa.

7. Repeat steps 1–6 for every bird.

8. When all the required kidneys have been removed from the birds, agitate them in the falcon and discard the PBSa. Repeat this process until the wash PBSa looks clear.

3.2 Isolation and Culture of Kidney Cells

1. Prior to processing the kidney tissues, prepare the digestion solution by diluting Trypsin-EDTA in a 3:1 ratio with PBSa. While kidneys are being extracted, pre-warm PBSa, Trypsin-EDTA solution and growth medium at 37 °C. In particular, ensure that the Trypsin-EDTA digestion solution is continuously maintained at 37 °C (even in between digestions).

2. Perform the next steps in a clean lab in a separate MBSC from the one used for kidney extractions.

3. Transfer the falcon tube with the freshly extracted kidneys inside a clean MBSC along with the sterile beaker, tweezers, Petri dish and two disposable scalpels.

4. Pour the contents of the falcon into a beaker before tipping one or two kidneys at a time onto the Petri dish. Using two scalpels remove the kidneys tissue from the organ membrane with gentle “sweeping” movements. Shred this kidney core finely teasing away obvious blood clots and white connective tissue.
5. Transfer the minced tissue into a sterile 500 ml baffled flask with approximately 100 ml of warm PBSa.

6. Once all the kidneys have been processed, rinse the tissue several times with approximately 100 ml PBSa until the supernatant runs clear. During each wash, discard the PBSa by gently swirling and allowing the fragments to settle for approximately 30 s (see Note 3).

7. Add 80 ml of warm diluted Trypsin-EDTA solution to drained tissue along with the magnetic stirrer bar and digest in a 37 °C incubator on the heated stirring platform, set to 36 °C, at a moderate speed for 4 min (see Note 4).

8. Allow the tissue to settle and pour the supernatant into a conical 1 l flask containing 100 ml of cold NBCS (see Note 5). Gently swirl the flask to ensure Trypsin-EDTA neutralisation in the serum.

9. Repeat steps 7 and 8 until no more tissue remains (see Note 6).

10. Position the metal gauze filter on top of the glass funnel with muslin and filter the cell suspension/NBCS mix collected in step 8 through this into a fresh sterile conical flask.

11. Distribute filtered suspension equally into sterile 250 ml centrifuge bottles and spin at 300 × g for 10 min to pellet the cells.

12. Carefully discard the supernatant from the centrifuge bottles and resuspend the cell pellets in warm growth medium, mixing enough to ensure a single cell suspension.

13. Pass the cell suspension through a 50 ml syringe connected to the Swinnex filter, collecting the filtrate in a fresh 250 ml wide-necked conical flask (see Note 7).

14. Using measuring cylinder, determine the volume of resulting cell suspension. Perform a cell viability count using trypan blue (see Note 8).

15. Following the cell count, dilute to required concentration in complete medium and place CK cells in culture flasks or plates in a 37 °C incubator to grow until the required confluency. Change the media 3 days after seeding to remove unattached cells and facilitate the formation of an even monolayer.

4 Notes

1. Wire mesh is obtainable from Locker Wire Weavers, www.wiremesh.co.uk.

2. Should any internal organ be punctured, stop work immediately, discard carcass into the appropriate waste stream and discard/clean tray.
3. During this step, some kidney cells may be lost but it is an effective way of excluding red blood cells from the final product.

4. Increasing the speed of the stirrer gradually during latter repeats ensures a thorough mechanical breakdown/digestion of the kidney tissues.

5. This NBCS volume is given for digestion of 5 pairs of kidneys and a maximum of 10 digestion steps. If by the end of tenth digestion, there is still significant amount of tissue left or in the case of more than 10 kidneys being used, the volume of NBCS should be increased correspondingly (10 ml NBCS per each additional digestion step).

6. During the first few steps, digestion occurs quite slowly while later, after the tissue bits are saturated with the Trypsin-EDTA solution, significantly higher numbers of cells are released.

7. Consecutive filtrations in steps 10 and 13 are aimed at removing any of the undigested tissue aggregates and larger cell clumps, correspondingly. By doing so, better quality of CK cell monolayers is achieved, which is essential for plaque assays.

8. During the cell count, red blood cells, which can be distinguished from the kidney cells by their size and shape, and dead blue cells should be excluded.

Acknowledgements

Elena Lokhman and Srijana Rai contributed equally to this work.

References

1. Maassab HF (1959) The propagation of multiple viruses in chick kidney cultures. Proc Natl Acad Sci U S A 45(7):1035–1039. https://doi.org/10.1073/pnas.45.7.1035

2. Tannock GA, Bryce DA, Paul JA (1985) Evaluation of chicken kidney and chicken embryo kidney cultures for the large-scale growth of attenuated influenza virus master strain A/Ann/Arbor/6/60-ca. Vaccine 3(3):333–339

3. Freshney RI (2011) Primary culture. In: Freshney RI (ed) Culture of animals cells: a manual of basic technique and specialized applications, edn. Wiley, New York, pp 163–186. doi:10.1002/9780470649367.ch11

4. Kendal AP, Kiley MP, Maassab HF (1973) Comparative studies of wild-type and "cold-mutant” (temperature-sensitive) influenza viruses: polypeptide synthesis by an Asian (H2N2) strain and its cold-adapted variant. J Virol 12(6):1503–1511

5. Parkin NT, Chiu P, Coelingh K (1997) Genetically engineered live attenuated influenza a virus vaccine candidates. J Virol 71(4):2772–2778

6. Seo SH, Collisson EW (1997) Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J Virol 71(7):5173–5177

7. Hughes CS, Jones RC (1988) Comparison of cultural methods for primary isolation of infectious laryngotracheitis virus from field material. Avian Pathol 17(2):295–303. https://doi.org/10.1080/03079458808436448

8. Maier HJ, Neuman BW, Bickerton E, Keep SM, Alrashedi H, Hall R, Britton P (2016) Extensive coronavirus-induced membrane rearrangements are not a determinant of pathogenicity. Sci Rep 6:27126. https://doi.org/10.1038/srep27126
9. Goldsmith CS, Tatti KM, Ksiazek TG, Rollin PE, Comer JA, Lee WW, Rota PA, Bankamp B, Bellini WJ, Zaki SR (2004) Ultrastructural characterization of SARS coronavirus. Emerg Infect Dis 10(2):320–326. https://doi.org/10.3201/eid1002.030913

10. Maier HJ, Hawes PC, Cottam EM, Mantell J, Verkade P, Monaghan P, Wileman T, Britton P (2013) Infectious bronchitis virus generates spherules from zippered endoplasmic reticulum membranes. MBio 4(5):e00801–e00813. https://doi.org/10.1128/mBio.00801-13

11. Bickerton E, Maier HJ, Stevenson-Leggett P, Armesto M, Britton P (2018) The S2 subunit of infectious bronchitis virus Beaudette is a determinant of cellular tropism. J Virol 92 (19). https://doi.org/10.1128/jvi.01044-18

12. Casais R, Dove B, Cavanagh D, Britton P (2003) Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol 77 (16):9084–9089. https://doi.org/10.1128/jvi.77.16.9084-9089.2003

13. Batra A, Maier HJ, Fife MS (2017) Selection of reference genes for gene expression analysis by real-time qPCR in avian cells infected with infectious bronchitis virus. Avian Pathol 46 (2):173–180. https://doi.org/10.1080/03079457.2016.1235258

14. Hennion RM, Hill G (2015) The preparation of chicken kidney cell cultures for virus propagation. Methods Mol Biol 1282:57–62. https://doi.org/10.1007/978-1-4939-2438-7_6