**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have checked the manuscript for errors.  
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].

We emailed The Journal of General Virology regarding permissions and received the following reply: “The copyright transfer agreement/licence to publish that was signed by the authors gives express permission for authors to reuse their own content, provided that the journal is acknowledged as the original source. No further permission is required.” We have uploaded a copy of this email and updated the figure legends.

3. Figure 2B: Please capitalize the first word in the labels on the x axis (i.e., Time).

This has been addressed.  
4. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

This has been addressed.  
5. Please provide an email address for each author.

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6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

This has been addressed.  
7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

This has been addressed.  
8. Please use centrifugal force (x g) for centrifuge speeds.

This was already the case.  
9. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

This has been addressed.  
10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

This has been addressed.  
11. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

This has been addressed.  
12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has been addressed.  
13. Line 86: Please specify the culture conditions.

This has been addressed.  
14. Line 94: Please mention the pore size of the filter.

This has been addressed.  
15. Line 100: What is used to titrate the chCD40L solution?

As described, the chCD40L is titrated using primary bursal cells. Briefly, the chCD40L is serially diluted and the cells cultured in the presence of the chCD40L dilutions. The lowest concentration where cell proliferation and viability is adequate should be used in the infection experiments. The text has been modified to clarify this in the protocol and discussion.  
16. Lines 129-130: Please add more details to this step. For instance, mention how animals are anesthetized and how proper anesthetization is confirmed. Please specify all surgical instruments used. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

No animals are anaesthetized. Animals are humanely culled and tissues removed at necropsy. Describing the culling steps is beyond the scope of our protocol. The labs that are trying to adopt this protocol will already have procedures in place to humanely cull their animals. In addition, legal methods of culling differ from country to country and it would be inappropriate to be prescriptive in our protocol. There are also multiple ways to remove the organ and the lab should use the protocols in place at their institution. The text has been modified to describe one such method if no protocols are in place.

17. Line 131: What volume of PBS is used to wash?

This has been addressed.  
18. Line 132: How large is the petri dish?

This has been addressed.  
19. Line 134: Please specify the instrument used to cut.

This has been addressed.  
20. Line 159: Please specify the volume of cell suspension and Trypan Blue solution used.

This has been addressed.  
21. Lines 187-192: Please add more details to your protocol steps. For instance, how are plaque assay or TCID50 assay performed? Please ensure that for all PCR, conditions and primers are listed. Alternatively, add references to published material specifying how to perform the protocol action.

A reference has been added.  
22. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

This has been addressed.  
23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

This has been addressed.  
24. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Can the editor please advise an appropriate endnote format to use? JoVE is not an option in Endnote X8  
25. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Can the editor please advise an appropriate endnote format to use? JoVE is not an option in Endnote X8  
  
**Reviewers' comments:**  
  
  
  
**Reviewer #1:**  
Manuscript Summary:  
Dulwich and coworkers describe the isolation of chicken B cells from the bursa of Fabricius for cell culture and infection with Infectious Bursal Disease Virus (IBDV). The protocol will be very useful for the study of primary chicken B cells and studies on several other immunosuppressive chicken viruses that cause major losses in poultry farming. Furthermore, this protocol may prove very useful for the isolation and culture of B cells of other avian species as monoclonal antibodies for positive or negative selection for B cell isolation are largely lacking for other avian species. The title and abstract are succinct. The protocol is detailed with all reagents needed and steps well explained and appears straightforward to allow successful implementation by other groups. The data is convincing with appropriate controls included and findings nicely illustrated.  
  
Major Concerns:  
None  
  
Minor Concerns:  
None

We thank the reviewer for their comments.  
  
**Reviewer #2:**  
Manuscript Summary:  
IBDV is an important immunosuppressive pathogen responsible for the Infectious Bursal Disease(IBD). The very virulent strain of IBDV is still difficult to grow in currently available celllines, which hampers the research progress in pathogenesis of IBDV. The authors describe the isolation of chicken primary bursal cells from the bursa of Fabricius, the culture and infection of the cells with IBDV, and quantification of viral replication. The addition of chicken CD40 ligand significantly increased cell proliferation and enhanced cell viability. Importantly, in additon to D78, a cell-culture adapted strain of IBDV, the very virulent strain UK661 replicated well in the ex vivo cell cultures. This model will be very helpful to the investigators working on the pathogenesis of IBDV. Overall, the article was well and clearly written and very informative to those working in this field. However there is a minor concern to be addressed.  
  
Major Concerns:  
No  
  
Minor Concerns:  
Lines 100-103. There is still much room in Step 5 for improvement. The purity of CD40L is a potential issue. It would be much better if the quantity of CD40L was more accurately determined with SDS-PAGE using a purified non-relevant protein of similar mass as a standard control for comparison, followed by the measurement of the protein band of interest with densitometry. Thus, the final concentration of CD40L in the culture medium could be calculated as microgram/mL instead of dilution. In this case, the different concentrations of CD40L purified from batch to batch could be properly diluted when CD40L was used in cell culture.

We thank the reviewer for their comments. We agree that the protein could be purified from the supernatant in the manner they describe. This is something that we could do going forward. For our current needs, simply concentrating the supernatant and adding it as a 1:20 -1:50 dilution to the culture media works adequately.   
  
  
**Reviewer #3:**  
Manuscript Summary:  
The manuscript described an interesting method to isolate chicken primary bursal cells from the bursa of Fabricius and then successfully infected the cells with different virulent IBDVs. The protocols were clearly described. The only concerns is that the origin of the important ingredient "chCD40L" that support the cell growing is complicated to obtain, which means it is still difficult for other researchers to follow the protocol even though the authors noted that phorbol 12-myristate 13-acetate could be used as well instead of chCD40.

We thank the reviewer for their comments. The chCD40L is available to labs upon the signing of an appropriate MTA. However, we have acknowledged the limitation of this in the discussion and provide a reference where PMA is used to stimulate the cells which the reader can try.  
  
  
**Reviewer #4:**  
Title: An ex vivo chicken primary bursal-cell culture model to study IBDV pathogenesis  
  
Review report  
The manuscript is well written and designed and has relevance in the field of infectious diseases in poultry. Following are my concerns that should be addressed in the revised manuscript.  
1. Bursal tissue contains heterogeneous cell population. So the characterisation of B-cells should be mentioned properly like markers, antibodies used. Although flowcytometry procedure in the discussion portion (line 271) mentioning the increased B-proliferation by the chCD40L stimulation has been indicated. But should be properly explained.

We thank the reviewer for their comments and we agree that the population of cells is heterogeneous. We have therefore been careful to use the term ‘bursal’ cell and not ‘B’ cell throughout the manuscript. Flow cytometry was used to characterise the cell population before and after chCD40L stimulation, using standard conditions, but we have not yet characterised the infected cell population with cellular markers. When staining with the chicken B cell marker anti-Bu-1 (SouthernBiotech), we found that 97% of our cultures stained positive following chCD40L stimulation. The text has been adjusted to reflect this. It is also important to determine whether the Bu-1 negative population are infected with IBDV, and we have added this statement to the manuscript text. This work is ongoing in the laboratory.   
2. It could have been more informative and conclusive if the primary bursal tissue cells infected with IBDV(untreated) would have been compared with the infected primary bursal tissue cells (treated with chCD40L) to demonstrate specific B-cell infection.

As shown in figure 1, the number of cells is low and the viability poor when the cells are not treated with chCD40L. We did not infect these cultures with IBDV as it would be difficult to make meaningful conclusions as the cell population is dying before infection and may be altering the expression of cellular genes. It might be interesting, however, to do these experiments in the future to determine whether the Bu-1 negative population are infected. However, as we have stated ‘bursal’ cell population in this manuscript, these experiments are beyond the scope of the current study.  
3. Line 57-62 Reference should be incorporated

This text has been removed as a result of revisions  
4. Line 76 Indicate type of cell

We have modified the text to state that the cells are primary bursal cells stimulated with chCD40L.  
5. Line 99 Provide molecular weight of protein. Also mention how supernatant containing protein is sterilised filtered.

The molecular weight of the protein is 55kDa (see Tregaskes et al., Dev & Comp Imm, 2005). The supernatant from the stably expressing cell-line is first concentrated and then passed through a 0.2 µm syringe filter to ensure no bacterial contamination is present.  
6. Line 107-108 indicate amount of calcium

HBSS supplemented with calcium is available to buy from ThermoFisher (cat no: 14060-040), but it does not specify the amount of calcium present in the buffer. We have provided JoVE with a materials list and include the catalogue number to help the reader.  
7. Line 113 50ul of insulin-transferin-sodium-slenite in how much amount of media.

This is for one 500ml bottle of IMDM. We have addressed this in the text.  
8. Line 140 Check spelling of "gentile"

This has been addressed.  
9. Line 142 Correct the sentence that "do no not"

This has been addressed.  
10. Line 147 Explain "ensure any missing"

‘Ensure any mixing’ refers to the fact that the cell suspension should be layered on top of the histopaque. We have removed this from the manuscript to avoid confusion.   
11. Line 163 Mention concentration of CD40L

This has been addressed.  
12. Line 172 Explain properly which type of cells are resuspended

This has been addressed.  
13. Line 174 Mention appropriate MOI

The appropriate MOI will depend on the experiment in question. If a multi-step growth curve is required, a low MOI is recommended. If a single step growth cure is required, a high MOI is recommended.