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# A protocol for lentiviral transduction and downstream analysis of intestinal organoids.

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| **Abstract:** | Intestinal crypt-villus structures termed organoids, can be kept in sustained culture three dimensionally when supplemented with the appropriate growth factors. Since organoids are highly similar to the original tissue in terms of homeostatic stem cell differentiation, cell polarity and presence of all terminally differentiated cell types known to the adult intestinal epithelium, they serve as an essential resource in experimental research on the epithelium. The possibility to express transgenes or interfering RNA using lentiviral or retroviral vectors in organoids has increased opportunities for functional analysis of the intestinal epithelium and intestinal stem cells, surpassing traditional mouse transgenics in speed and cost. In the current video protocol we show how to utilize transduction of small intestinal organoids with lentiviral vectors illustrated by use of doxycylin inducible transgenes, or IPTG inducible short hairpin RNA for overexpression or gene knockdown. Furthermore, considering organoid culture yields |

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| | minute cell counts that may even be reduced by experimental treatment, we explain how to process organoids for downstream analysis aimed at quantitative RT-PCR, RNA-microarray and immunohistochemistry. Techniques that enable transgene expression and gene knock down in intestinal organoids contribute to the research potential that these intestinal epithelial structures hold, establishing organoid culture as a new standard in cell culture. |
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**A protocol for lentiviral transduction and downstream analysis of intestinal organoids.**

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Lentivirus, Retrovirus, Organoid, Intestine, Transduction, Stem cell, Lgr5, Endoplasmic reticulum, ER stress, unfolded protein response.

**SHORT ABSTRACT:**

In this video protocol we give a step by step explanation of lentiviral transduction in organoids of primary intestinal epithelium and of processing and downstream analysis of these cultures by quantitative RT-PCR, RNA-microarray and immunohistochemistry.

**LONG ABSTRACT:**

Intestinal crypt-villus structures termed *organoids*, can be kept in sustained culture three dimensionally when supplemented with the appropriate growth factors. Since organoids are highly similar to the original tissue in terms of homeostatic stem cell differentiation, cell polarity and presence of all terminally differentiated cell types known to the adult intestinal epithelium, they serve as an essential resource in experimental research on the epithelium. The possibility to express transgenes or interfering RNA using lentiviral or retroviral vectors in organoids has increased opportunities for functional analysis of the intestinal epithelium and intestinal stem cells, surpassing traditional mouse transgenics in speed and cost. In the current video protocol we show how to utilize transduction of small intestinal organoids with lentiviral vectors illustrated by use of doxycylin inducible transgenes, or IPTG inducible short hairpin RNA for overexpression or gene knockdown. Furthermore, considering organoid culture yields minute cell counts that may even be reduced by experimental treatment, we explain how to process organoids for downstream analysis aimed at quantitative RT-PCR, RNA-microarray and immunohistochemistry. Techniques that enable transgene expression and gene knock down in intestinal organoids contribute to the research potential that these intestinal epithelial structures hold, establishing organoid culture as a new standard in cell culture.

**INTRODUCTION:**

The intestinal epithelium is one of the most rapidly proliferating bodily tissues, which has caused it to attract wide interest from research on cancer and stem cells. In 2009 a technique was published to generate long lasting cultures of small intestinal crypts in matrigel, conserving a 3 dimensional structure[1]. These structures, termed intestinal organoids, can be cultured using standard techniques, with surrounding medium supplemented with a number of defined growth factors, including the Bmp-signaling pathway inhibitor noggin (Nog), the Wnt-signaling pathway enhancer rspondin 1 (Rspo1) and epidermal growth factor (Egf) all found to enhance intestinal proliferation[2-4].

Organoids surpass traditional cancer cell lines in the aspects that they are non-mutated, have maintained stem cell hierarchy, display intact cellular polarization and exhibit differentiation into all cell lineages found in the nascent small intestinal epithelium. Since they can be transduced to carry transgenes or RNA interference constructs[5], they are used to study specific genetic elements, outweighing experiments using transgenic mice in facets of cost and speed. Transgenic expression in organoids can be performed using either murine retroviral or lentiviral vectors[6,7]. Due to the limitations of murine retroviruses, capable of transducing mitotic cells exclusively[8], lentiviral transduction is more frequently used for cells that are difficult to infect, such as organoids.

Virally transduced and stably expressing transgenic organoids can be used for a multitude of downstream analyses, including quantitative RNA analyses and immunohistochemistry. Taken together, culture of organoids from primary intestinal epithelial cells has evolved into a routine technique that is easy to implement without specific laboratory requirements, and has become the

novel standard in cell culture in research on the intestinal epithelium.

Techniques of viral transduction and subsequent downstream analysis in organoids are tedious to perform and to aid organoid experiments generated this video protocol, showing methods for lentiviral transduction of cultured organoids. We additionally show how correct processing of organoids can increase yield and therefore enhance performance of downstream analysis using RNA techniques or immunohistochemistry. In the protocol, organoids that are derived from small intestinal crypts were exclusively used, although the techniques described may be applied to colonic organoids as well.

**PROTOCOL:**

**1. Preparation of polyethylenimine (PEI) as transfection reagent.**

1.1 Dissolve approximately 150 mg of PEI into 100 ml of $H_2O$.

1.2 Adjust solution to pH 7.4 by adding HCl until solution becomes clear and stir until completely dissolved. This may take between 10 and 60 minutes and add water to an end concentration of 1 mg/ml.

1.3 When clear, filter the PEI solution through sterile 0.22 µm filter and store in a -80 °C freezer in aliquots of 5 ml.

**2. Production of lentiviral particles**

*Day 1:*

2.1. Split HEK293T cells to 60-80% confluency in 162 $cm^2$ flask or large petri dish in cell line culture medium (DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 2 mM glutamine supplement).

*Day 2:*

2.2. Prepare DNA transfection solution containing 45 µg of total plasmid DNA by adding together lentiviral packaging vectors (7 µg of pVSVg; 5 µg of pRSV rev; 13 µg of pMDL) and 20 µg of lentiviral plasmid encoding the gene of interest or shRNA of interest. Adjust to a volume of 1 ml using DMEM.

2.3. Prepare PEI transfection solution by adding 90 µl of 1 mg/ml PEI to 930 µl of DMEM and incubate for 5 minutes at room temperature.

2.4. Add DNA transfection solution to PEI solution. Vortex or invert a number of times and incubate for 5 minutes at room temperature to obtain DNA transfection solution.

2.5. Drip 2 ml of the DNA transfection solution onto the HEK293T cells and incubate for 4 hours in a humidified cell culture incubator on 37 °C.

2.6. After 4 hours, refresh the culture medium to remove PEI. It is not necessary to wash cells before adding new medium.

Note: PEI is cytotoxic and incubation times longer than 4 hours may cause harm to the HEK293T cells.

Day 4:

2.7. 'Replace supernatant with new culture medium. Keep supernatant (containing virus); this will be used in step 2.10.

2.8. Put supernatant in 15 ml flask. To remove dead cells, centrifuge for 5 minutes at 500 x *g*.

2.9. Push supernatant through 0.45 µm filter using a large 60 ml syringe. Store overnight at 4 °C.

Day 5:

2.10. Collect the second batch of supernatant; centrifuge and filter as in step 2.8-2.9.

2.11. Pool the supernatants from step 2.9 and 2.10 in ultracentrifuge tubes and centrifuge at 50.000 x *g* in an ultracentrifuge for 90 minutes.

2.12. Take out capsules containing the ultracentrifuge tubes very carefully and put into a laminar flowhood, remembering the orientation of the tube inside the centrifuge.

2.13. Open capsule holding ultracentrifuge tube and decant medium carefully in such a fashion that the pellet is on the upper side of the tube. Since viral pellets may be difficult to visualize, remember on what side of the tube a pellet will have formed. Take a micropipette and remove last bit of medium while taking care not to agitate the opaque brown pellet that is visible on the side of the bottom of the ultracentrifuge tube.

2.14. Resuspend this pellet in 500 µl of organoid culture medium supplemented with 10 mM nicotinamid, 10 µM Chir99021, 10 µM Y27632 and 8 µg/ml polybrene. Resuspension in this medium is important since high titer virus is used to tranduct organoids directly.

2.15. **Optionally**, freeze the virus in this medium aliquotted in two batches of 250 µl in -80 °C.

## 3 Lentiviral transduction of organoids.

*Day 0*

3.1 Split a full 0.95 $cm^2$ well of organoids two days prior to transduction into a new well, aiming to obtain approximately 50 small organoids. Split organoids according to previously published protocol[1] (Figure 3A, B).

3.2 Supplement organoid culture medium with 10 µM Chir99021 and 10 mM nicotinamide to obtain cystic hyper proliferative crypts (Figure 3C).

Note: Cystic crypts will develop best when freshly split organoids are grown in the presence of Chir99021.

*Day 2*

3.3 Harvest organoids by pipetting up and down the matrigel and medium, thereby disrupting the mixture with a p1000 micropipette. Place the mixture in a 15 ml tube.

3.4 Disrupt further using Pasteur pipet in which the distal opening has been decreased by melting. Centrifuge organoids to pellet for 5 minutes at 100 x *g*.

Note: Dependent on the size of the centrifuge, use a different centrifuge speed. It is necessary to find the exact speed in which disrupted organoids are separated from the mixture.

3.5 Remove the supernatant and add 500 µl of pre-warmed 1x trypsin (similar to 0.25% trypsin). Resuspend organoids in trypsin and incubate 3 minutes in a 37 ºC water bath.

3.6 Inactivate trypsin by adding 3.5 ml of cell line culture medium (DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and glutamine suppletion). Centrifuge for 5 minutes at 500 g.

3.7 Remove the supernatant to leave pellet in approximately 20 µl of medium. Transfer the organoids in this last small amount of medium into a well on a 48-wells plate.

3.8 Add high titer lentivirus in transduction medium as described in step 2.14, resuspend and continue to step 3.10. When encountering low transduction efficacy, step 3.9 may be added.

Note: For efficient transduction, typically add one aliquot of 250 µl of high titer lentivirus to one well of organoids. This represents 50% of all harvested lentivirus from a single production as described in step 2 of this protocol.

3.9 **Optionally**, to enhance transduction efficacy, perform spinoculation by putting the 48 well plate containing organoids in a pre-warmed centrifuge on 32 °C and rotate at 600 x *g* for 1 hour.

3.10 Put organoid-virus mixture in culture incubator and incubate for 1 hour at 37 °C in a culture incubator to allow transduction.

3.11 **Optionally**, to further enhance transduction efficacy, incubate organoids for additional 3 hours at 37 °C in a culture incubator.

3.12 Add 1 ml of organoid culture medium and resuspend the organoid-virus mixture, transfer into a microcentrifuge tube and centrifuge in a microcentrifuge for 5 minutes at 850 x *g* to pellet organoids.

3.13 Remove the supernatant and resuspend the pellet in 20 µl of ice-cold matrigel. Since the material solidifies when it becomes warmer, use pipette tips that are chilled by pipetting up and down ice cold PBS for a number of times.

3.14 Put the droplet in the middle of a well in a 48 wells plate and incubate in culture incubator at 37 °C for 15 minutes to solidify.

3.15 After 15 minutes, carefully add 250 µl of organoid culture medium supplemented with 10 mM nicotinamide, 10 µM Chir99021 and 10 µM Y27632. Small disrupted organoid fragments will form into small cystic organoids within 24 hours.

*Day 5*

3.16 Refresh medium and supplement with a selection antibiotic (for puromycin, use 4 µg/ml).

*Day 7*

3.17 Replace medium for standard organoid culture medium, supplemented with selection antibiotic.

**Note: Budding will be complete 2-3 weeks after Chir99021 withdrawal.** After selection, organoids may be grown without selection antibiotic.

## 4 Organoid RNA preparation for Quantitative RT-PCR or microarray

4.1 For RNA preparation, use a commercial kit according to manufacturer's protocol. Remove the medium from organoids and add 350 µl of buffer RLT, supplemented with β-mercaptoethanol straight on the dome of matrigel containing organoids. Resuspend the material in RLT by pipetting using p1000 micropipette.

4.2 Perform further RNA prep according to manufacturer's protocol.

4.3 **Optionally**, increase RNA yield by amplification using Ovation Pico WTA system, requiring initial input of 50 µg total RNA according to manufacturer's protocol.

4.4 **Optionally**, for quality control prior to RNA microarray, run samples on a 2100 bioanalyzer using an eukaryote total RNA nano chip, aiming for an RNA integrity number (RIN) of 8.5 or more (Figure 5) according to manufacturer's protocol.

## 5 Processing organoids for paraffin embedding and immunohistochemistry.

5.1 Prior to initiation of organoid embedding pre-warm an aluminum block with holes fitting 12 mm diameter glass tubes in incubator at 70 °C to keep paraffin liquid.

5.2 Take a single well with full-grown organoids and remove the medium, leaving the embedded organoids intact.

5.3 Add 1 ml of 4% paraformaldehyde in PBS straight to well and fix at 4 °C anywhere from 1 hour to overnight.

5.4 Replace paraformaldehyde with 1 ml of ice cold PBS. **Optionally**, keep fixed organoids in PBS up to 1 week at 4 °C after this step.

5.5 Resuspend fixed organoids in 1 ml of PBS and place into glass vial.

5.6 Let organoids sink to bottom for 1 minute, decant PBS and replace with 70% ethanol in which a couple of droplets of eosin solution are dissolved to enable visualization of organoids throughout the embedding process.

5.7 Leave organoids in 70% ethanol at room temperature. After 30 minutes, remove 70% ethanol by carefully decanting, being able to visualize the organoids by eye because of slight pinkish eosin color. Replace embedding solution with 96% ethanol.

5.8 Repeat step 5.7, each time replacing the embedding solution with the next one. Pass organoids through the following solutions subsequently: 70% ethanol, 90% ethanol, 96% ethanol, 100% ethanol, 100% ethanol, xylene, xylene.

5.9 Decant the last xylene wash and pour paraffin into the tube. Put the tube immediately in the pre warmed aluminum block at 70 °C for 30 minutes and replace paraffin with new clean paraffin.

5.10 Pour paraffin off and pipet organoids into the paraffin block mold using a pre-warmed Pasteur pipet with large opening. Keep Pasteur pipet warm using a Bunsen burner. Place all organoids in the paraffin block mold in a small layer of liquid paraffin.

5.11 As much as possible, try to manipulate all organoids towards the center of the paraffin block mold using a warmed dissection needle. Keep dissection needle warm using Bunsen burner.

5.12 When localization of organoids in the mold is satisfactory, chill mold slightly to solidify the paraffin layer.

5.13 Finish the block by pouring more paraffin on top and add a standard histological embedding cassette.

## REPRESENTATIVE RESULTS:

*Organoid lentiviral transduction*

The technique of organoid transduction using lentiviral particles depends on correct handling of organoids prior and during transduction. Organoids (Figure 3A) were cultured and they were disrupted into single crypts (Figure 3B). As previously reported, these single crypts, when cultured in the presence of the GSK3 inhibitor Chir99021 became cystic crypts[9] (Figure 3C). Subsequently organoids were trypsinized to allow penetration of virus particles to single cells. When transducing cells with lentiviral particles, a number of methods may be tried to enhance transduction efficacy such as spinoculation or prolonged incubation. High titer PGK-eGFP lentivirus was used to enable visualization of transduction efficacy by fluorescent microscopy. Transduction efficacy of organoids with this plasmid was high and approached 100% (Figure 4E, F). Improving efficacy using spinoculation (Figure 4A, B) or prolonged incubation with lentiviral particles (Figure 4C,D) did therefore not yield additional value.

*RNA extraction*

Next organoids for RNA extraction were grown. Full-grown organoids were harvested that were subjected to gamma irradiation or control treatment to show reduced RNA integrity (Figure 5). Upon irradiation with 6 Gy, RNA is degraded and compared to control treated organoids, the RNA integrity number (RIN) is reduced.

*Immunohistochemistry on paraffin embedded organoids*

After incubating organoids for 2 hours in culture medium supplemented with BrdU, organoids in formaline were fixed and processed for immunohistochemistry (Figure 6). Using mouse anti BrdU, proliferative cells could be observed in crypt-segments of organoids and not in the differentiated compartment.

**Figure 1: Schematic of lentivirus production for organoid transduction.** As written in protocol part 2, in this schematic, the most critical steps of virus production are represented with protocol step numbers and timing.

**Figure 2: Schematic of lentiviral transduction of organoids.** As written in protocol part 3, in this schematic, the most critical steps of organoid transduction are represented with protocol step numbers and timing.

**Figure 3: Organoids prior to and during transduction.** Normally growing organoids (A) are split into densely growing small organoids (B) that become cystic after incubation with Chir99021 for a number of days (B). Upon dissociation of these organoids using trypsin, single cells and small clumps of cells remain (D) that are transduced subsequently. Scale bar 100 µm.

**Figure 4: Transduction of organoids using lentiviral expression vectors.** Brightfield images (A,C,E,G) and fluorescent images (B,D,F,H) from organoids that were transfected with either PGK-eGFP lentivirus (A –F) or control lentivirus (G,H). eGFP expression in organoids after lentiviral transduction using both spinoculation and extended incubation (A,B), spinoculation only (C,D) or no additional steps to increase transduction efficacy (E,F), compared to control vector transduced organoids, that do not express eGFP (G,H). Note that using PGK-eGFP lentiviral construct, transduction efficacy is not greatly increased by additional steps. Scale bar

100 µm.

**Figure 5: representative result of RNA preps from organoids on bioanalyzer.**
Note strong demarcation of bands representing high RNA integrity in lane 2-4 and smear around bands on lanes 5-7 representing log RNA integrity. RNA integrity number (RIN) of bands 2-4 is 8.7, 9.2 and 9 respectively, whereas the RIN of bands 5-7 is 6.4, 6.6 and 5.5.

**Figure 6: Immunohistochemical analysis of formalin fixed paraffin embedded organoids.**
Formalin fixed paraffin embedded 4 µm section of organoids cultured in the presence of BrdU for two hours and fixed subsequently. Section is stained with anti-BrdU antibody. Scale bar 100 µm.

**Table 1:**
**Culture medium composition**

**DISCUSSION:**
The current video protocol describes lentiviral transduction of organoids from primary intestinal epithelium and downstream analysis of these organoids using quantitative RNA techniques and immunohistochemistry.

Lentiviral transduction is often performed in adherent or floating cells in culture plates. Since the three-dimensional structure of organoids renders them difficult to penetrate by viral particles, a number of methods to increase efficacy are used. Pretreatment of organoids using Chir99021 increases proliferation and thereby viability after transduction. It is important to maintain small cell clusters after trypsinization, since single cells have decreased survival. In contrast to viral transductions using murine retrovirus, lentiviral transductions are more efficient and ordinarily do not require spinoculation, a technique known to increases transduction efficacy in ES cells[10]. When using murine retroviruses or when lentiviral transduction yields low efficacy, spinoculation can optionally be utilized to increase transduction rate. We do not regularly assess virus titer, since all available virus from a single round of production for transduction of organoids derived from maximally two 0.95 $cm^2$ wells was used. In addition, generally use antibiotic selection for removal of non-transduced cells. Assessment of viral titer may however be of value when encountering problems with transduction efficacy.

Identical to adherent cells, the level of selection antibiotic can increase the number of viral integrations and thereby expression of the transgene. Use high-level puromycin (10 µg/ml) to obtain high transgenic expression and effective knockdown, but when organoids exhibit signs of toxicity, lower levels may be used (minimally 1 µg/ml). In addition, alternative selection antibiotics may be used or selection may be omitted, although transduction is likely to be incomplete and this may not result in long-term stable expression. Furthermore, since lentiviral integration in the genome is heterogeneous throughout a population of cells, expression of the transgene may be subdue to changes after culturing cells for a prolonged time. This may result from selective pressure by expression of the transgene. Although antibiotic selection limits these changes, they may remain, especially in the case of constitutive expression of transgenes. This disadvantage may be overcome by single cell cloning of organoids, but this technique is time consuming. Lentiviral transduction can be performed with a wide variety of plasmids that result

in either stable or inducible expression of transgenes. These transgenes are usually expressed from ubiquitous promoters, such as the CMV or the PGK promoter and may result in supernatural expression. Similar to transgenic overexpression in cancer cell lines or model animals, caution is warranted interpreting results from overexpression.

For experiments that require RNA, the low number of viable cells normally grown in a single 0.95 $cm^2$ well (standard surface area of 48 well) in contrast to adherent cells that can easily be grown in large flasks is the rate-limiting factor for quality and multitude of downstream analysis. We find that normally, a single well of approximately 50 full grown organoids yields between 0.4 μg and 1 μg total RNA, being sufficient for quantitative RT-PCR and RNA microarray without further amplification. Certain treatment regimens or genetic alterations can reduce the RNA content significantly however. A first step to increase total RNA is pooling of multiple wells.

For paraffin embedding of organoids, it is critical to obtain sufficient material in order to visualize organoids during the process. Addition of minute amounts of eosin to fixed organoids allows visualization of organoids during the whole process, but is not required for normal embedding and may be omitted when this step interferes with further analysis. Usually, paraffin-embedding cassettes have holes in them to allow flow of fixative and process solutions to the tissue in the cassette.

Lentiviral organoid transduction and preparation for downstream standard techniques increases scientific potential of these cultures and raises the standard in cell culture to three-dimensional techniques from primary intestinal epithelium. The range of downstream experiments to be performed is however not limited to techniques described in the current protocol and may encompass all techniques performed on cell lines or mice, given the amount of cellular material generated by culture is sufficient. In research on specific genetic elements and their function in the intestinal epithelium, homologous recombination techniques in model animals, most notably mice, remain the gold standard. Cultures of organoids do not contain mesenchymal or immunological niche cells and cultured crypts are ever expanding, contrasting the situation found *in vivo*. Nontheless, these cultures have raised the quality of *in vitro* findings greatly and taking into account the myriad downstream techniques that can be performed, organoid culture has and will greatly enhance research on the intestinal epithelium.

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**DISCLOSURES:**
The authors have nothing to disclose.

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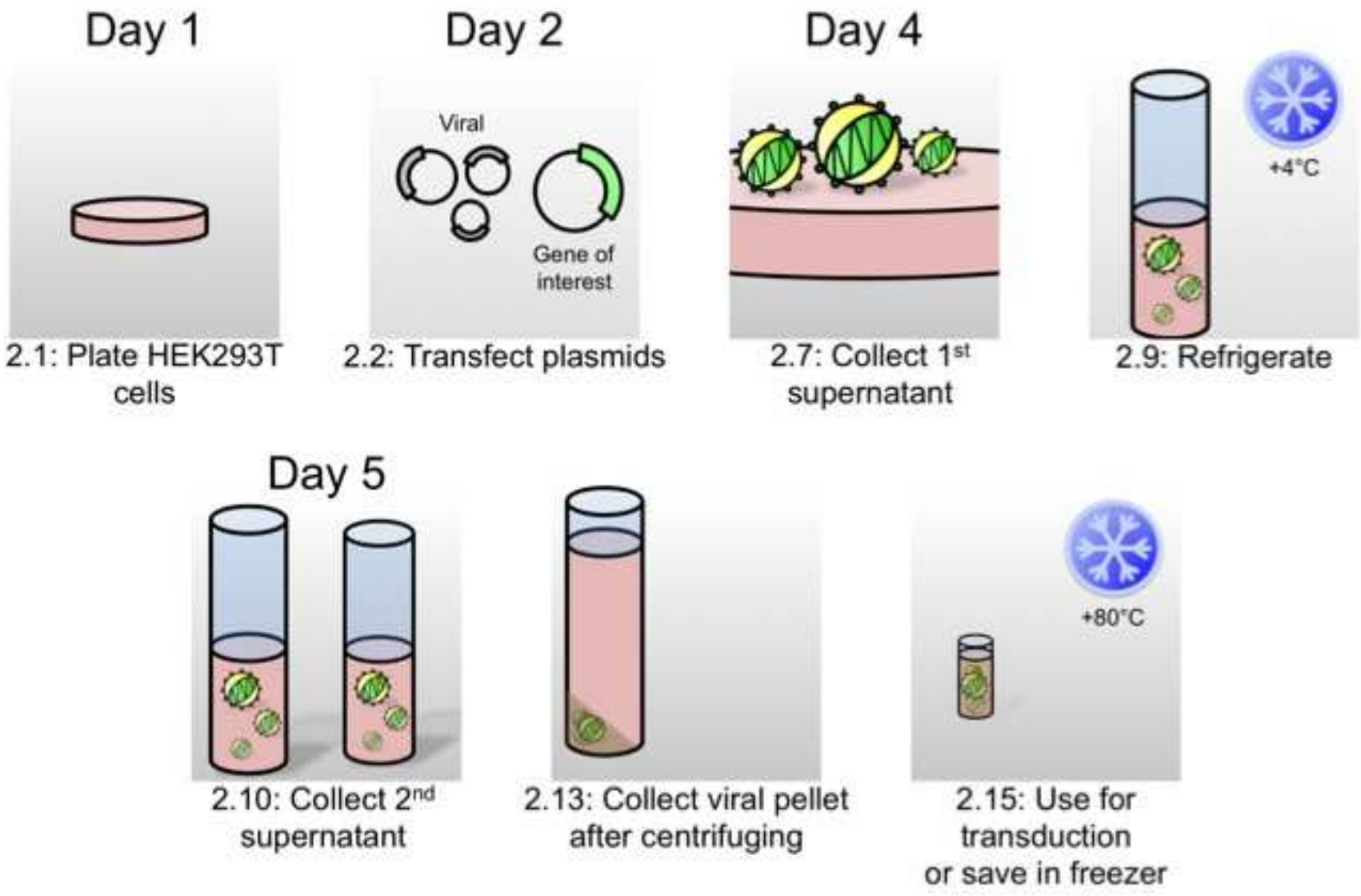

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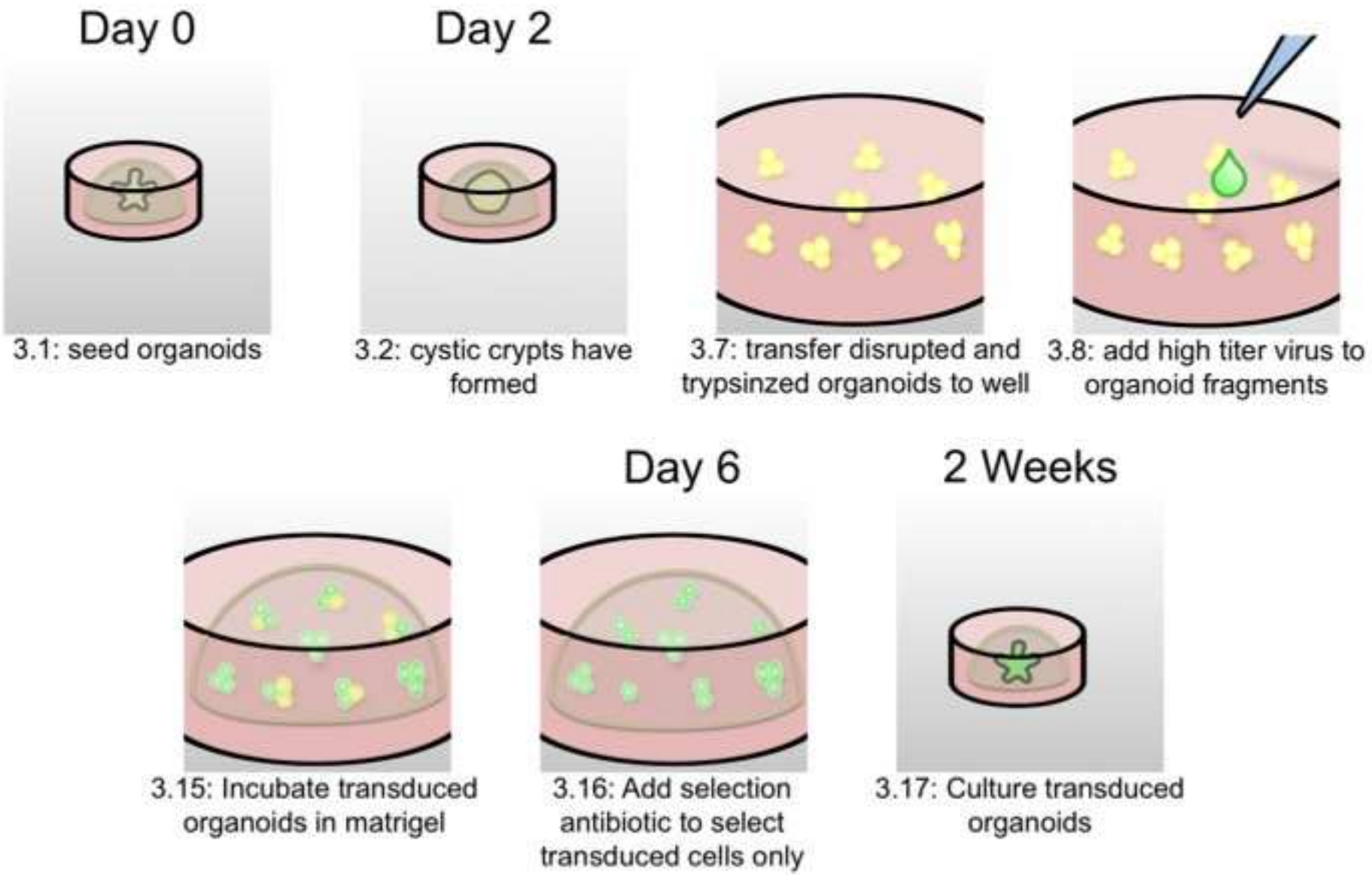

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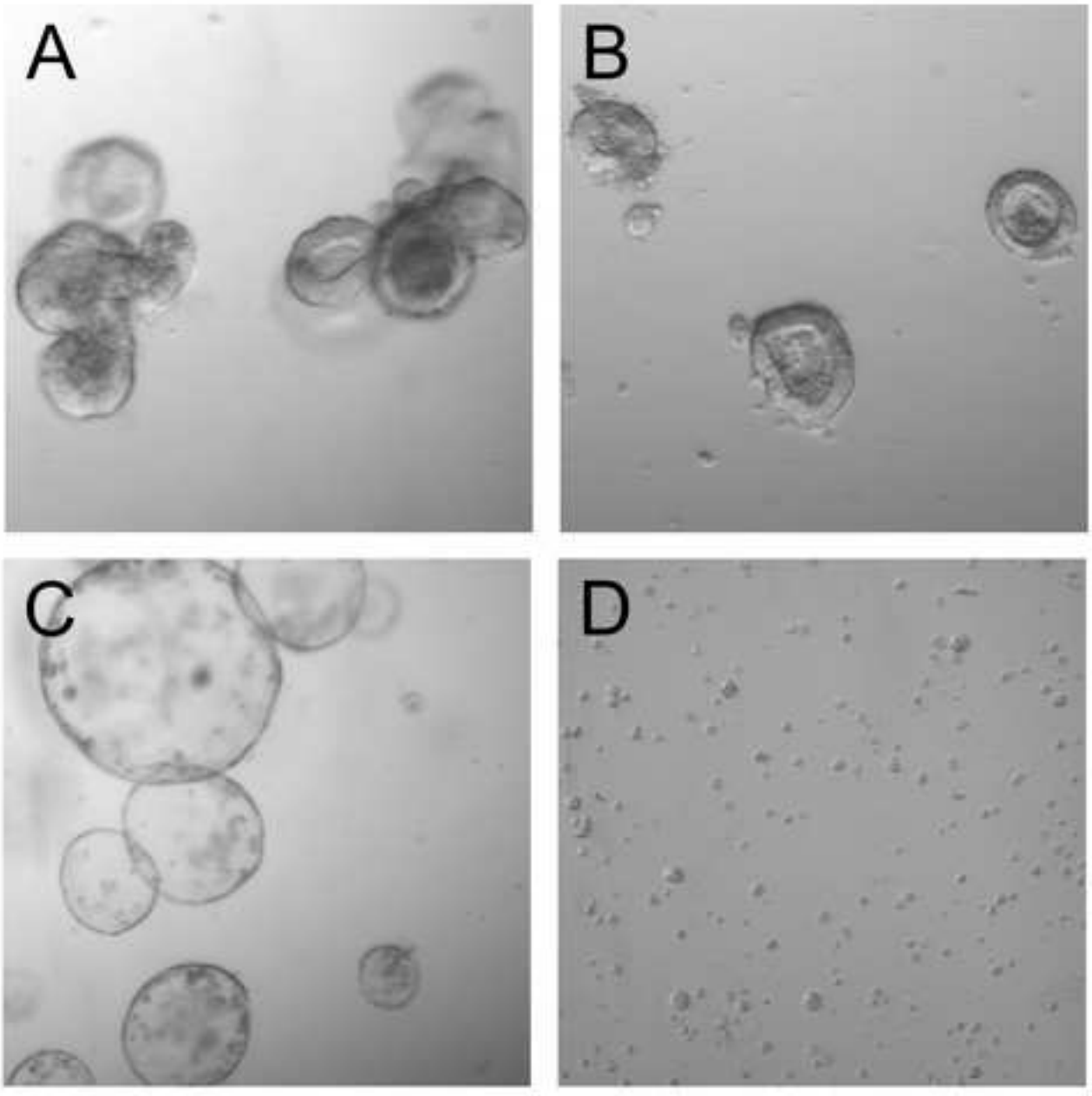

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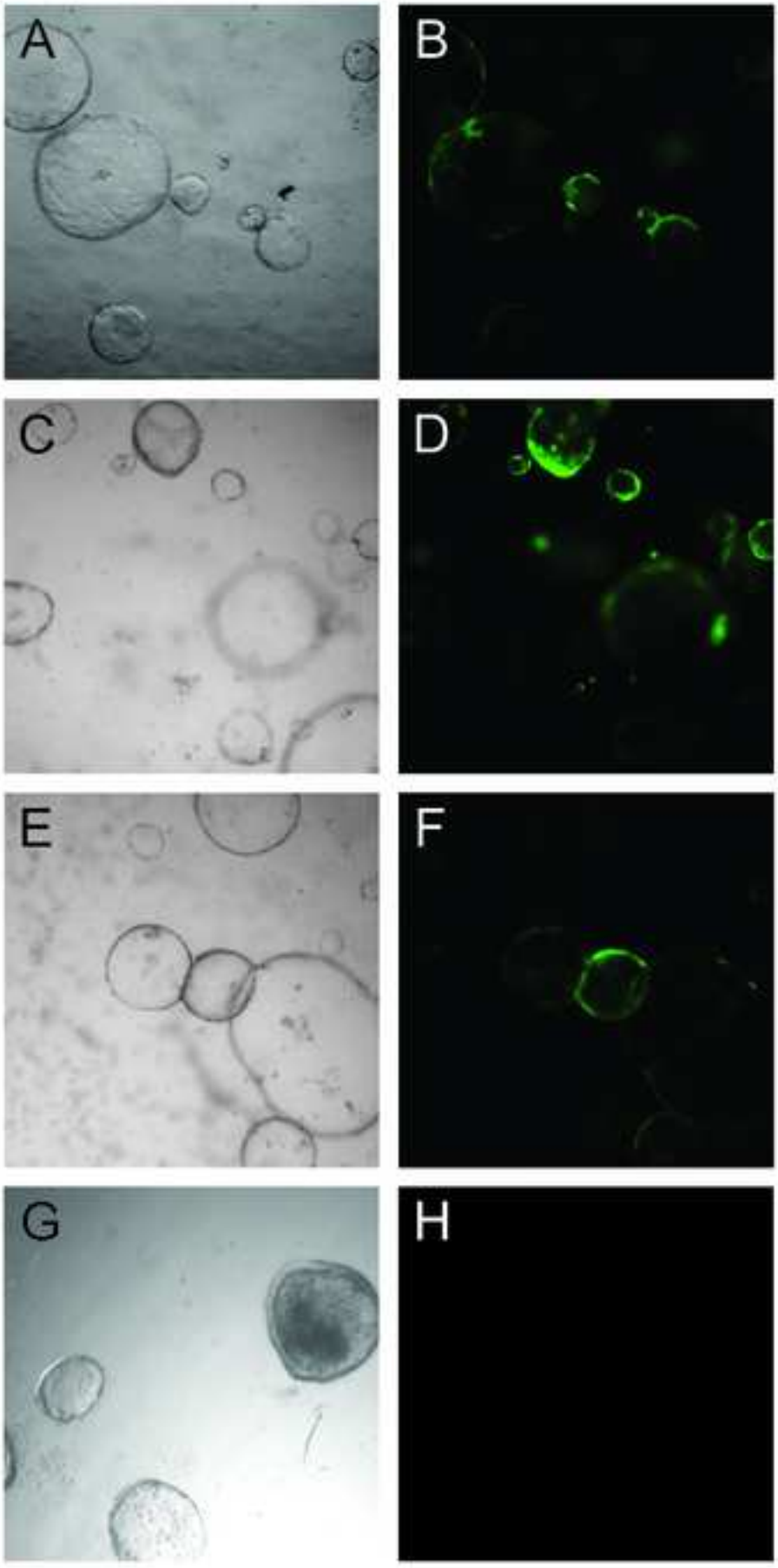

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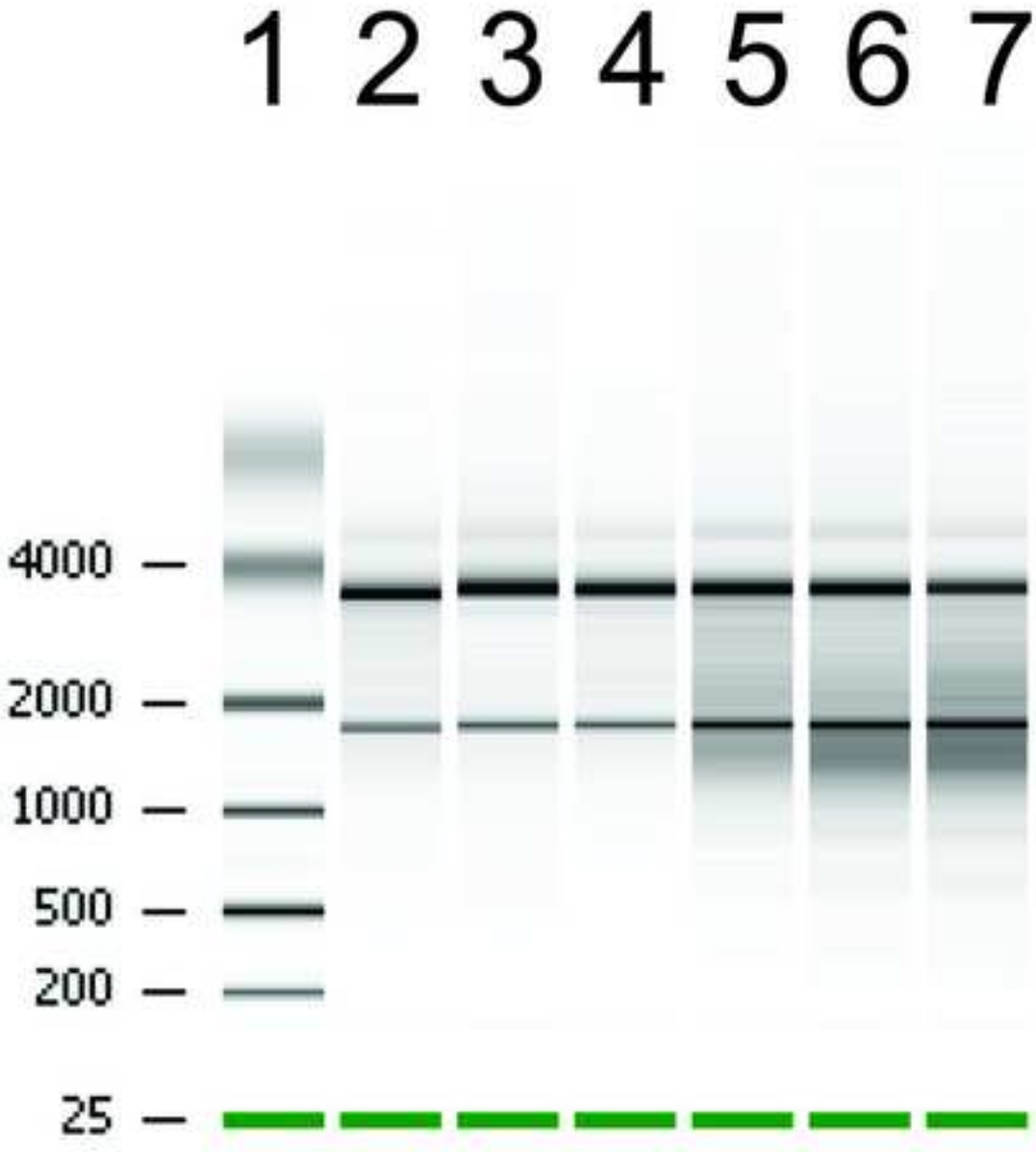

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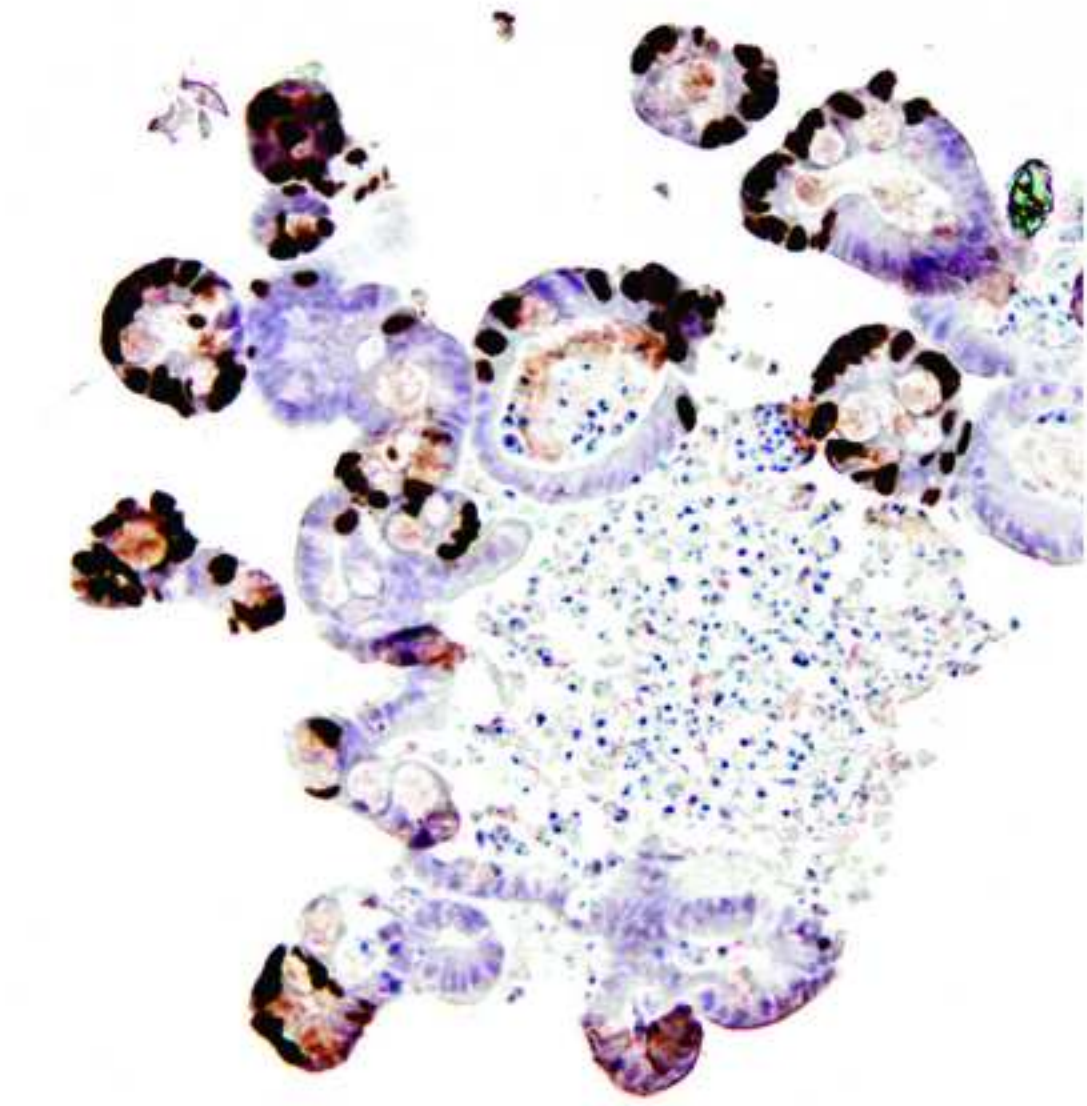

Table 1
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| | **Basic medium** |
|---|---|
| Cell line culture medium | DMEM |
| Organoid culture medium | Advanced DMEM F12 |

| **Additions** |
| --- |
| 10% FCS<br>1% penicillin/streptomycin<br>2mM Glutamine |
| HEPES<br>1% penicillin/streptomycin<br>1x glutamax<br>1% N2 supplement<br>2% B27 supplement<br>125 nM n-acetyl cysteine<br>mouse Egf (50 ng/ml)<br>10% Nog-Fc conditioned medium (equivalent to 100 ng/ml)<br>10% Rspo1-Fc conditioned medium (equivalent to 500 ng/ml) |

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table 2.xlsx

| Reagent | Company | Cat. No. |
|---|---|---|
| Polyethylene imine | polysciences | 23966-2 |
| DMEM medium | Lonza | BE12-614F |
| Fetal calf serum | Lonza | DE14-801F |
| Penicillin-streptomycin | Invitrogen | 15140-122 |
| Glutamin | Invitrogen | 25030-024 |
| matrigel | BD | BD 356231 |
| Advanced DMEM-F12 | Gibco | 12634-010 |
| N2 | Invitrogen | 17502-048 |
| B27 | Invitrogen | 17504-044 |
| N-acetyl cysteine | Sigma | A9165-1G |
| mouse Egf | Invitrogen | PMG8045 |
| Hepes 1M | Invitrogen | 15630-056 |
| glutamax 100x | Invitrogen | 35050-038 |
| Chir 99021 | axon | 1386 |
| Y27632 | Sigma | Y0503-5MG |
| polybrene | Sigma | 107689 |
| nicotinamide | Sigma | N0636 |
| Trypsin | Lonza | BE02-007E |
| puromycin | sigma | P 7255 |
| Rneasy mini kit | Qiagen | 74106 |
| b-mercaptoethanol | Merck | 8,057,400,250 |
| Ovation Pico WTA system | NuGen | 3300-12 |
| paraformaldehyde | Sigma | 252549-1L |
| glass vial conical 12mm x 75mm 5ml | VWR | LSUKM12 |
| Eosin Yellowish | VWR | 1,159,350,025 |

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Title of Article: A protocol for lentiviral transduction and downstream analysis of intestinal organoids

Author(s): J. van Lidth de Jeude, P. Montenegro-Miranda, J. Vermeulen, GR van den Brink, J. Heijmans

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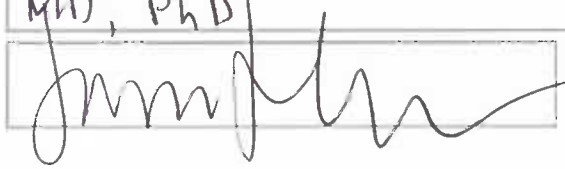
Signature:  Date: 18-July-2014

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Dear Editor,

After your and the reviewers extensive reviews, we have altered our manuscript for publication in JoVE. We feel the current manuscript has improved significantly and we thank you and the reviewers for all comments. Pleas find below a point by point answer on all questions and amendments suggested in all review.

On behalf of my co-authors,

Sincerely,

Jarom Heijmans

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 extensively proofread the manuscript and found it fitting to our convenience.*

2. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). step 3.6.

*This was done*

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Matrigel" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language. Matrigel is used 8 times.

*As we wrote previously, matrigel is a difficult term to avoid. Although we have altered other brand names in the text in the previous version, we were not able to come up with a generic for matrigel. It would seem cumbersome to alter matrigel into sentences as "solidifying extracellular matrix" throughout the text. We therefore prefer the term matrigel remains used in the manuscript, due to lack of a suitable alternative. If such an alternative would be apparent, we would not doubt to alter the word in the text.*

4. Please revise in the Discussion: "as low as 1 $\mu$ g/ml"

*This was altered.*

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes in detail the transduction of intestinal organoid culture cells using lentiviral vectors. It includes how to expand and produce the lentiviral particles for transduction and how to handle transduction of organoids. Organoid culturing has previously been described but the manuscript does a good job of detailing important additions to improve transduction. An important point they make is that making the organoids cystic through the addition of nicotinamide, Chir99021 and Y27632 is crucial for efficient transduction. They also highlight the preparation of organoids for paraffin embedding for IHC. Both of these techniques will be invaluable to have video documentation as these are nuanced techniques that are often overlooked but crucial for high quality research outcomes. Additionally they describe how to harvest RNA for downstream analysis.

Major Concerns:
I have no major concerns other than there is new publication coming out that describes organoid transduction using retroviruses, which is similar but I believe this manuscript goes beyond that publication.

Minor Concerns:

I have no concerns other than the few minor edits listed below.

Additional Comments to Authors:
The use of the term "stove" as opposed to "incubator" seems to be a dutch translation error, unless this is a British English term I am not familiar with. In the long abstract Line 57 change "RNA interference" to "interfering RNAs". Line 59 delete "in specific".Line 161 "Presumable" should be deleted. Line 163 should read "brown pellet that is visible". Line 315 teh special character is missing from GSK3. Line 335 change "groing" to "growing". Line 397 shoudl read "RT-PCR". Line 406 should read "...have holes in them to allow..."Line 407-409- I am not sure the point of these last few sentences having not seen the video but I beleive the point they trying to make is that normal cassettes cannot be used and only blocks described here can contain the organoids as described here. I would clarify and delete or modify these last few sentences.

*We have altered or adapted the manuscript based on the reviewers comments.*

Reviewer #2:
In the last couple of years, intestinal derived organoid cultures have become the gold standard assay to study intestinal stem cell biology in vitro. They have proven to be superior to 2D serum based cultures as they more closely recapitulate crucial in vivo biological processes, i.e hierarchical organization and multi lineage differentiation potential. In addition, they provide a cost effective system that allow for genetic and pharmacological manipulation. In this video protocol, de Jeude et al describe a detailed methodology for standard molecular practice such as IHC, lentiviral transduction and RNA extraction. Although of limited novelty, this manuscript is informative and will certainly facilitate future research in the area of organoid culture systems.

Minor comments:

The authors could start and briefly describe the culture method and then dive into the different methodology used in the manuscript. Now it starts with the preparation of transfection reagent that I would suggest to bring at the end where reagents are described.
Throughout the manuscript, the authors are encouraged to adopt some consensus on the form. Notes are sometimes written in bold or not and "optionally". That should be consistent.
Reagent concentrations should be clearly defined, what is 1x Trypsin.
The RIN quantification could be substantiated with a qPCR efficiency of a house keeping gene.

*Based on comments of reviewer 2, we have adopted a consequent style and layout throughout the manuscript*

Reviewer #3:
Manuscript Summary:
van Lidth de Jeude et al. describe a protocol of transduction of organoids, as well as protocols for RNA extraction and organoids processing for immunohistochemistry. The paper is well written and detailed protocols on experimental procedures with small intestinal organoids are of interest for a broad range of researchers. The methods are clearly described and scientifically sound.

Major Concerns:
no major concerns

Minor Concerns:
- Throughout the manuscript (and title), I would mention that these methods apply to small intestinal organoids.

*We have adopted the manuscript to this comment, although not the title. We have added a sentence to clarify this to the introduction.*

- In general, I would recommend that the authors move part of the explanation from the discussion section to the introduction. That will improve the readability of the protocols. I.e. explanation about the function of Chir99021, Y27632..

*We have reread our article to find a suitable spot for this addition, but we have not added it to the introduction of the manuscript, since we fear it might interefere with readability.*

- Protocol, step 1: mention that PEI is very cytotoxic

*We have added a note after 2.6. to clarify this point.*

- Step 2.2: rename for consistency the term DNA transfection solution, since this is what you obtain after addition of the PEI solution (step 2.4)

*We have altered this in the text.*

- Step 2.5: incubate for 4 hours in…

*We have altered this in the text.*

- Step 2.5 (and throughout manuscript): replace 'stove' with incubator

*We have altered this in the text.*

- Step 2.6: are cells washed?

*We have added a sentence to 2.6 clarifying that it is necessary to wash cells in this step.*

- Step 2.7-2.9: 'Replace supernatant with new culture medium. Keep supernatant (containing virus); this will be used in step 2.10. To remove death cells, spin down for 5 minutes at 500g, filter through a 0.45um strainer and store at 4degrees.

*We have altered steps 2.7-2.9 based on these comments*

- Step 2.10: Collect second batch of supernatant and repeat steps 2.7-2.9
- Step 2.11: Pool the supernatants (step 2.9 and step 2.10)..

*These steps were altered.*

- Step 2.12: I would mention in this step rather than in 2.13 that it is important to mark (maybe better then to remember) the tube to determine its the orientation in the ultracentifuge

*We have altered 2.12 and 2.13 and merged this paragraph with 2.14*

- Step 2.17: Did the authors perform a tittering step to check for transduction efficacy? Although there is a puromycin selection step, this step could reduce batch to batch variation.

*We have added a sentence to explain why we do not use tittering steps in the discussion.*

- Step 3.1: specify well size

*This was specified.*

- Step 3.5: what concentration trypsin is used?

*This was added to 3.5*

- Step 3.6: for consistency please use g instead of rpm

*This was altered*

- Step 3.9/3.10: It is not clear whether step 3.9 is (optionally) added to the protocol or performed instead of step 3.10.

*We have clarified this with an extra sentence in 3.8.*

- Step 3.11: is something taken out before resuspending or is 1ml organoid culture medium added to the whole mixture, and then mixed? Please clarify

*This was clarified.*

Reviewer #4:

This manuscript written by van Lidth de Jeude et al thoroughly explains a protocol for lentiviral transduction of organoids. Furthermore, the authors explain several techniques to process the organoids for further analysis. The manuscript is well written and the step-by-step protocols are easy to follow. This manuscript allows others to easily implement this relative new technique, which is currently replacing standard cell line culture in the field of intestinal epithelial research; other types of tissue will soon follow.

I have no major comments, just a few minor suggestions that are more a matter of taste. Therefore, I recommend publication of this manuscript with or without the implementation of my suggestions.

Minor suggestions:

1. Make an overview comparing organoids to cell lines and list the pros and cons of both techniques (maybe include mouse models). This way, a relative inexperienced user immediately sees the discrepancies and can choose accordingly. As I am not familiar with the editorial layout of the JOVE website I suggest the editors decide the appropriate location of such an overview within the article.

*We would prefer the article to be of methodological value, without discrediting other techniques. A pro-con list of characteristics of cell lines versus organoids may do this. We have therefore not added it to our manuscript.*

2. Make some sort of small flow-chart figure that gives an overview of the complete protocol including the duration of each step.

*We have entered a schematic of the protocols 2 and 3 with protocol step numbers and timing, that will hopefully add to the comprehensibility of the protocol.*

3. On page 10, line 408, it says, "To our knowledge, no cassettes exist that have small enough holes to prevent from falling out, but this may be an easy alternative." I think it should be "….to prevent organoids from falling out….."

*We have removed this entire remark.*

4. In the "representative results" section I would recommend to include subtitles for the specific techniques performed, for example "RNA extraction" and "IHC processing". This, to improve the structure of the section.

*We have added these subheadings to the manuscript.*