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Comprehensive Experimental Workflow for Filovirus Infection Studies in iPSC-derived and Primary-derived Human Gut Organoids Part 1

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Elizabeth Flores[1], Elizabeth Flores[2]

[1]Center for Regenerative Medicine (CReM), Boston University Chobanian & Avedisian School of Medicine and Boston Medical Center, Boston, MA, USA.;
[2]National Emerging Infectious Diseases Laboratories (NEIDL), Boston University Chobanian & Avedisian School of Medicine, Boston, MA, USA.

Elizabeth Yvonne Flores

Elizabeth Flores

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Disclaimer

All procedures involving human induced pluripotent stem cells (hiPSCs) were approved by the Boston University Institutional Review Board. Parental iPSC lines, including the BU1CG line harboring a CDX2/GFP reporter (SPC2-ST-B2 clone), were previously described and are available through the CReM iPSC Repository (https://crem.bu.edu/cores-protocols/). The BU1CG line is derived from a healthy male donor and exhibits a normal 46XY karyotype.

Abstract

Experimental Workflow:
This workflow outlines the step-by-step experimental pipeline for studying Ebola virus (EBOV) and Marburg virus (MARV) infection in human intestinal and colonic organoid systems. It integrates organoid differentiation from induced pluripotent stem cells (hiPSCs) and primary human colonic tissue, standardized infection procedures, functional assays, and downstream immunofluorescence and flow cytometry analyses. Each section provides detailed methods and reagents to ensure reproducibility in BSL-4-compatible systems and support comparative studies of filovirus pathogenesis in 3D human models.

Sections:
Protocol 1: Differentiation and Culture of Human iPSC-derived Intestinal and Colonic Organoids and Culture of Primary Colonic Organoids
This step defines the foundational organoid culture platform that supports all downstream viral infection and analysis assays.
Protocol 2: Standardized Infection of Human Gut Organoids with Ebola (EBOV) and Marburg (MARV) Viruses
This describes the infection workflow including inoculation, timing, containment, and MOI setup.

Materials

2. Materials and Reagents

2.1 Cells
- Human iPSC lines (e.g., BU1CG, CDX2/GFP reporter)
- Primary human colonic tissue-derived cells (PCOs)

2.2 Matrices

Matrix	Source	Catalog #
Growth Factor Reduced Matrigel	Corning	356230
Matrigel Basement Membrane Matrix	Corning	354234

2.3.a Small Molecules 26 Growth Factors

Name	Source	Catalog #	Working Concentration
SB431542	Tocris	1614	3 µM
Dorsomorphin	Stemgent	04-0024	3 µM
CHIR99021	Tocris	4423	3 µM
Recombinant human FGF10	R26D Systems	345-FG-025	10 ng/mL
Recombinant human KGF	R26D Systems	251-KG-010	10 ng/mL
Recombinant human BMP4	R26D Systems	314-BP	10 ng/mL
Retinoic acid	Sigma	R2625	0.1 µM
Y-27632 dihydrochloride	Sigma	1254	10 µM
Dexamethasone	Sigma	D4902	50 nM
cAMP	Sigma	B7880	0.1 mM
IBMX	Sigma	I5879	0.1 mM
Recombinant Human FGF4	Thermo Fisher	H3570	10 ng/mL
Trypsin-EDTA (0.05%)	Thermo Fisher	25300-120	as needed
Defined Fetal Bovine Serum	NC0652331	as indicated
Recombinant human Noggin	R26D Systems	6057NG025	500 ng/mL
Recombinant human EGF	R26D Systems	236EG200	100 ng/mL
Recombinant Human R-Spondin 1 Protein	R26D Systems	4645-RS-025	100 ng/mL
N2 Supplement	Thermo Fisher	17502-048	1x
B27 Supplement	Invitrogen	12587-010	1x
GlutaMAX	Invitrogen	35050061	1x

2.3.b Antibodies 26 Flow Cytometry Reagents

| Target | Vendor | Catalog # |

| --- | --- | --- |
Anti-human CD47 PerCP/Cy5.5 Conjugate	BioLegend	323110
Anti-human CD26 PE Conjugate	BioLegend	302705
Calcein Blue	Life Technologies	C1429

2.4 Media Recipes

iPSC-derived Organoid Differentiation Media

Medium	Components
IM+CHIR	RPMI-1640 + 2% FBS + CHIR99021 3 µM
IM+CK	RPMI-1640 + 2% FBS + CHIR99021 3 µM + KGF 10 ng/mL + FGF4 10 ng/mL
Hindgut Medium	IMDM 75% + Ham's F12 25% + B27 + N2 + Retinoic Acid 0.1 µM
cSFDM	DMEM/F12 + HEPES + GlutaMAX + B27 + N2 + CHIR99021 3 µM + KGF/FGF7 10 ng/mL
CK+DCI	cSFDM + CHIR99021 3 µM + KGF/FGF7 10 ng/mL + Dexamethasone 50 nM + cAMP 0.1 mM + IBMX 0.1 mM

Primary-Derived Colonic Organoid Media

Medium	Components
Splitting Medium	500 mL DMEM/F12, 10% FBS, 1% Penicillin/Streptomycin
DF20	500 mL DMEM/F12, 20% FBS, 1% Penicillin/Streptomycin
R-spondin selection medium	500 mL Advanced DMEM + 50 mL FBS + 5 mL Penicillin/Streptomycin + 5 mL Glutamax + 1.5 mL Zeocin
R-spondin collection medium	500 mL DMEM/F12 + 5 mL Glutamax. Note no Penicillin/Streptomycin
R-spondin freezing medium	5 mL selection medium + 3 mL FBS + 2 mL DMSO
WNT selection medium	500 mL Advanced DMEM + 50 mL FBS + 2 mL G418 (100mg/mL). Note no Penicillin/Streptomycin.
WNT collection medium	500 mL Advanced DMEM + 50 mL FBS. Note no Penicillin/Streptomycin
WNT Freezing medium	5 mL WNT selection medium + 3 mL FBS + 2 mL DMSO
Basal Growth Medium (BGM)	DMEM/F12 + Primocin 100 ng/mL + B27 + N2 + Noggin 500 ng/mL + R-Spondin 100 ng/mL + EGF 100 ng/mL

Materials and Reagents:

Component	Specification / Source	Purpose / Notes
Human gut organoids	Distal HCOs, proximal HIOs, and primary-derived gut organoids	Maintained in 12-well plates embedded in Matrigel
Matrigel	Basement membrane matrix (Corning, pre-chilled)	3D scaffold for organoid culture and re-embedding

Cell Recovery Solution (CRS)	Corning	500 µL per well; preserves organoid integrity during harvesting
Phosphate-buffered saline (PBS)	Sterile	For rinsing and washing organoids
Organoid culture media	CKDCI, CKDI, IMCK, or primary-derived gut organoid media	CKDCI: distal HIOs; CKDI: distal-cAMP HIOs; IMCK: proximal HIOs; primary-derived gut organoid media (see table)
EBOV and MARV stocks	Purified from cell culture supernatants via ultracentrifugation through 20% sucrose cushion	Viral titers quantified by TCID_50 assay
P1000 pipette tips	Standard, distal end cut	Minimizes mechanical disruption during transfer of Matrigel droplets
1.5 mL microcentrifuge tubes	Sterile	Collection and centrifugation of organoid suspensions
Centrifuge	Capable of 200 × g	Pelleting organoids for medium exchange and infection preparation

Before start

1. Culture in CB/RA medium (CHIR99021 + rhBMP4 + Retinoic Acid). Add Retinoic Acid freshly to the medium before use.
2. Medium changes are required every other day.

Protocol 1: Differentiation of hiPSCs into Human Intestinal and Colonic Organoids and Culture of Primary Colonic Organoids

1 **Authors:** Elizabeth Yvonne Flores et al.

Abstract

2 This protocol describes two distinct workflows: A) Differentiation of human induced pluripotent stem cells (hiPSCs) into intestinal (HIO) and colonic (HCO) organoids, including the Day 15 sorting of CDX2-GFP^+^ gut progenitors and maintenance of organoids. B) Thawing, expansion, and maintenance of primary-derived colonic organoids (PCOs) from healthy adult donors. Reproducibility is ensured through standardized media formulations, defined growth factor conditions, and the use of independent biological replicates.

Materials and Reagents

3 Cells
- Human iPSC lines (e.g., BU1CG, CDX2/GFP reporter)
- Primary human colonic tissue-derived cells (PCOs)

4 Matrices

Matrix	Source	Catalog #
Growth Factor Reduced Matrigel	Corning	356230
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5 Small Molecules and Growth Factors

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CHIR99021	Tocris	4423	3 µM

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	Recombinant human EGF	R&D Systems	236EG200	100 ng/mL
	Recombinant Human R- Spondin 1 Protein	R&D Systems	4645-RS-025	100 ng/mL
	N2 Supplement	Thermo Fisher	17502-048	1x
	B27 Supplement	Invitrogen	12587-010	1x
	GlutaMAX	Invitrogen	35050061	1x
	Gentle Cell Dissociation Reagent	StemCell Technologies	07174	as per manufacturer
	Ascorbic Acid	Sigma	A4403	50 µg/mL

	Cell Recovery Solution	Corning	354253	as per manufacturer
	Paraformaldehyde	Electron Microscopy Sciences	19208	4%
	ReLeSR	StemCell Technologies	05873	as per manufacturer
	StemDiff Definitive Endoderm Kit	StemCell Technologies	05110	as per manufacturer
	Accumax	Sigma	A7089	as per manufacturer

6 Media Recipes

	Medium	**Components**
	IM+CHIR	RPMI-1640 + 2% FBS + CHIR99021 3 µM
	IM+CK	RPMI-1640 + 2% FBS + CHIR99021 3 µM + KGF 10ng/mL + FGF4 10 ng/mL
	Hindgut Medium	IMDM 75% + Ham's F12 25% + B27 + N2 + Retinoic Acid 0.1 µM
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	CK+DCI	cSFDM + CHIR99021 3 µM + KGF/FGF7 10 ng/mL +Dexamethasone 50 nM + cAMP 0.1 mM + IBMX 0.1 mM

7 Antibodies & Flow Cytometry Reagents

	Target	**Vendor**	**Catalog #**

	Anti-human CD47 PerCP/Cy5.5 Conjugate	BioLegend	323110
	Anti-human CD26 PE Conjugate	BioLegend	302705
	Calcein Blue	Life Technologies	C1429

8 Primary-Derived Colonic Organoid Media

	Composition of Culture Medium for Primary-Derived Gut Organoids
	Splitting Medium: 500 mL DMEM/F12, 10% FBS, 1 % Penicillin/Streptomycin
	DF20: 500 mL DMEM/F12, 20% FBS, 1% Penicillin/Streptomycin
	R-spondin selection medium: 500 mL Advanced DMEM + 50 mL FBS + 5 mLPenicillin/Streptomycin + 5 mL Glutamax + 1.5 mL Zeocin
	R-spondin collection medium: 500 mL DMEM/F12 + 5 mL Glutamax. Note noPenicillin/Streptomycin
	R-spondin freezing medium: 5 mL selection medium + 3 mL FBS + 2 mL DMSO
	WNT selection medium: 500 mL Advanced DMEM + 50 mL FBS + 2 mL G418(100mg/mL). Note no Penicillin/Streptomycin.
	WNT collection medium: 500 mL Advanced DMEM + 50 mL FBS. Note noPenicillin/Streptomycin

WNT Freezing medium: 5 mL WNT selection medium + 3 mL FBS + 2 mL DMSO

Equipment

9 - Tissue culture incubator (37°C, 5% CO_2)
- Class II biosafety cabinet
- Centrifuge with swinging-bucket rotor
- Flow cytometer

Methods

10 **Section A: iPSC-Derived Human Intestinal Organoids (HIOs) and Human Colonic Organoids (HCOs)**

11 **4.1 iPSC Maintenance**
Culture hiPSCs on growth factor-reduced Matrigel in mTeSR1.
Passage at 70–80% confluency using ReLeSR.
Confirm morphology and pluripotency markers.

12 **4.2 Differentiation to HIOs**
Day 0 – Definitive Endoderm Induction
Dissociate hiPSC colonies using Gentle Cell Dissociation Reagent.
Plate 2 × 10^6^ cells/well in mTeSR1 + 5 µM Y-27632 (ROCK inhibitor).
Differentiate using StemDiff Definitive Endoderm Kit.
Confirm CXCR4^+/c-Kit^+^ population via flow cytometry.
Refer to 2.3b Antibodies 6 Flow Cytometry Reagents.

13 **Day 3 – Hindgut Specification**
Passage 1:3 onto 2D Matrigel-coated plates.
Treat with DS/SB43 + Y-27632 for 24 h, then DS/SB43 alone for 48 h.

14 **Day 6-15 – Intestinal Patterning**
Culture in CB/RA medium (CHIR99021 + rhBMP4 + Retinoic Acid). Add Retinoic Acid freshly to the medium before use.
Medium changes are required every other day.

15 **Day 15: CDX2-GFP^+^ Sorting, Seeding, and Lineage Specification**
Cell Preparation and Sorting of CDX2-GFP+ Gut Progenitors: Collect Day 15 cells and dissociate them into single cells or small clusters using 0.05% Trypsin-EDTA for no longer than 5 minutes at 37°C. Immediately quench the enzymatic reaction by adding an equal volume of medium containing fetal bovine serum (FBS). Centrifuge the cell

suspension at 200 × g for 5 minutes, aspirate the supernatant, and resuspend the pellet in FACS buffer (e.g., PBS + 2% FBS). Pass the cell suspension through filtered FACS tubes with cell-strainer caps to remove aggregates. Add Calcein Blue AM to identify live cells. Prepare FACS collection tubes containing IM+CHIR medium supplemented with 10 µM Y-27632. Sort CDX2-GFP^+^ gut progenitors by fluorescence-activated cell sorting (FACS) directly into the chilled collection medium.
Centrifuge sorted cells (200 × g, 5 min, 4°C), resuspend in cold Growth Factor Reduced Matrigel (Corning) at 2,000–5,000 cells per 50 µL 3D matrigel dome. Plate domes on pre-warmed 12-well plates, polymerize at 37°C for 30 min, then overlay with appropriate medium:
- Intestinal organoids (HIOs): IM+CK medium + Noggin 500 ng/mL, + R-Spondin 100 ng/mL, + EGF 100 ng/mL + CHIR99021 3 µM.
- Colonic organoids (HCOs): cSFDM medium + CHIR99021 3 µM + KGF/FGF7 10 ng/mL + Dexamethasone 50 nM + cAMP 0.1 mM + IBMX 0.1 mM.
Include Y-27632 for 24 h post-sort, then remove. Feed every 3–4 days.

16 **Day 15 Onward: Long-Term Culture**
Maintain HIOs and HCOs in their respective media with medium changes every 3–4 days. Mature organoids for 14–21 days before experimental use. Passage or cryopreserve as needed.

17 **Section B: Primary Cell-Derived Colonic Organoids (PCOs)**
PCOs were provided by Dr. Ryan B. Corcoran (Massachusetts General Hospital) and derived from healthy adult donors. Unlike iPSC-derived organoids, PCOs do not undergo differentiation or sorting; they are maintained and expanded directly.

18 **4.4 Thawing and Recovery**
Thaw organoids rapidly in a 37°C water bath (1–2 min), transfer to a 15 mL tube with pre-warmed selection medium, centrifuge (200 × g, 5 min), and resuspend in fresh selection medium. Embed in 50 µL 3D Matrigel domes and overlay with Basal Growth Medium (BGM).

19 **4.5 Maintenance and Passaging**
Replace medium every 3–4 days. To passage, place plates on ice, add Cell Recovery Solution (CRS, 1 mL/well), incubate 15 min, then collect and incubate on ice for 1 h. Centrifuge (200 × g, 5 min), digest with TrypLE (5 min, 37°C), and mechanically dissociate with a 20G needle. Neutralize with Splitting Medium, centrifuge, resuspend in fresh Matrigel, and replate domes.

20 **4.6 Quality Control and Replication**
All organoid derivations were performed in at least three independent differentiations or passages per donor line. Morphology was confirmed microscopically and epithelial identity by immunofluorescence for CDX2, EPCAM, and MUC2. HIOs, HCOs, and PCOs were used for infection experiments only after confirmation of mature epithelial differentiation.

Quality Control

21 Flow cytometry at definitive endoderm stage (CXCR4^+/c-Kit^+^).
Morphology: uniform cystic structures.
Confirm absence of contamination and consistent differentiation efficiency.

Reproducibility Recommendations

22 Use ≥2 independent iPSC lines and ≥2 donor-derived PCO lines.
Perform ≥3 independent differentiations or expansions per line.

References

23 Flores, Elizabeth Y et al. "Human-induced Pluripotent Stem Cell-derived Gut Organoids Recapitulate Regional Specific Genetic Programs and a Role for cAMP in Lineage Specification." *Cellular and molecular gastroenterology and hepatology* vol. 19,9 (2025): 101534. doi:10.1016/j.jcmgh.2025.101534

24 Mithal, Aditya et al. "Generation of mesenchyme free intestinal organoids from human induced pluripotent stem cells." *Nature communications* vol. 11,1 215. 10 Jan. 2020, doi:10.1038/s41467-019-13916-6

Protocol 2: Standardized Infection of Human Gut Organoids with Ebola (EBOV) and Marburg (MARV) Viruses

25 **Authors:** Elizabeth Yvonne Flores et al.

Abstract

26 This protocol describes the controlled infection of iPSC-derived human intestinal organoids (HIOs), human colonic organoids (HCO) and primary-derived gut organoids with Ebola virus (EBOV) or Marburg virus (MARV), enabling quantitative and qualitative evaluation of viral replication dynamics and host cellular responses. All infection experiments with EBOV and MARV were conducted under BSL-4 containment in compliance with institutional and federal biosafety regulations. Organoids are first harvested from Matrigel and subjected to gentle dissociation and preparation steps to preserve structural integrity prior to exposure to viral inocula of defined multiplicity of infection (MOI). Following viral exposure, organoids are re-embedded in 3D Matrigel matrices to maintain tissue architecture, facilitating downstream analyses including histological, immunofluorescence, flow cytometry, and transcriptomic assays. Mock-

infected controls are processed in parallel to account for any perturbations arising from handling or culture manipulations. This methodology provides a robust and reproducible platform for assessing viral infectivity, investigating dose-dependent responses, and interrogating mechanistic host-pathogen interactions in physiologically relevant 3D human intestinal tissue models.

Materials and Reagents

27

	Component	Specification / Source	Purpose / Notes
	Human gut organoids	Distal HCOs, proximal HIOs, and primary-derived gut organoids	Maintained in 12-well plates embedded in Matrigel
	Matrigel	Basement membranematrix (Corning, pre- chilled)	3D scaffold for organoid culture and re-embedding
	Cell Recovery Solution (CRS)	Corning	500 µL per well; preservesorganoid integrity during harvesting
	Phosphate-buffered saline (PBS)	Sterile	For rinsing and washing organoids
	Organoid culture media	CKDCI, CKDI, IMCK, orprimary-derived gut organoid media	CKDCI: distal HIOs; CKDI: distal-cAMP HIOs; IMCK: proximal HIOs; primary-derived gut organoid media (see table)
	EBOV and MARV stocks	Purified from cell culture supernatants via ultracentrifugation through20% sucrose cushion	Viral titers quantified by $TCID_{50}$ assay
	P1000 pipette tips	Standard, distal end cut	Minimizes mechanical disruption during transfer of Matrigel droplets

	1.5 mL microcentrifuge tubes	Sterile	Collection and centrifugation of organoidsuspensions
	Centrifuge	Capable of 200 × g	Pelleting organoids for medium exchange and infection preparation

Methods

28 **Step 1 — Preparation of Organoids for Infection**

1. Aspirate culture medium from each well while preserving Matrigel droplet integrity.
2. Add 500 µL chilled CRS per well and incubate at 4°C for 30 minutes.
3. Transfer Matrigel droplets to 1.5 mL microcentrifuge tubes using a P1000 pipette tip with the distal end removed.
4. Rinse wells with 500 µL PBS to recover residual organoids and combine with the initial suspension.
5. Centrifuge at 200 × g for 5 minutes. Aspirate supernatant carefully, leaving minimal medium to avoid pellet loss.
6. Resuspend organoids in 1 mL of the appropriate culture medium (CKDCI for distal HIOs, CKDI for distal-cAMP HIOs, IMCK for proximal HIOs).

29 **Step 2 — Viral Stock Preparation**

7. Purify EBOV and MARV stocks from cell culture supernatants via ultracentrifugation through a 20% sucrose cushion.
8. Determine viral titers using the tissue culture infectious dose 50 (TCID_50) assay.
9. Process mock-infected controls in parallel, including dissociation, incubation, Matrigel re-embedding, media exchange, and inactivation, but without viral exposure.

30 **Step 3 —Determination of Multiplicity of Infection (MOI)**

10.Dissociate organoids from three representative wells into single-cell suspensions using enzymatic digestion.
11. Count cells to determine the average cell number per well to normalize for organoid size and density.
Calculate infectious units required: Infectious units = Average cell number per well × MOI × Number of wells
12. Dilute the viral stock in the appropriate organoid medium to achieve the desired MOI. Standard infections are performed at MOI = 10; additional MOIs (0.1, 10, 50, 100) may be used to evaluate dose-dependent responses.

31 **Step 4 —Viral Infection Procedure**

13. Add the calculated viral volume to the organoid suspension and incubate at 37°C for 1 hour.
14. Wash organoids once with 1 mL PBS.
15. Resuspend organoids in fresh 3D Matrigel and replate into 12-well plates.
16. Incubate at 37°C for 30 minutes to allow Matrigel polymerization.
17. Overlay with 1 mL of appropriate organoid medium and return plates to the incubator for downstream analyses.

Quality Control and Reproducibility

32

	Problem	Possible Cause	Recommended Solution
	Organoids dissociate orcollapse during harvesting	Excessive mechanicalstress or high centrifugation speed	Use cut pipette tips, keepsamples on ice, centrifuge at 200 × g
	Low infection efficiency	Inaccurate MOI calculation, diffusion barriers	Verify cell counts, optimize viral mixing, consider higher MOI for 3Dorganoids
	Matrigel does not polymerize properly	Temperature too low or delayed plating	Keep Matrigel and organoids at 37°C for atleast 30 min before adding medium
	High cell death in mock controls	Handling stress or prolonged manipulation	Minimize time on ice, handle droplets gently,reduce pipetting steps

Protocol references

1. Flores, Elizabeth Y et al. “Human-induced Pluripotent Stem Cell-derived Gut Organoids Recapitulate Regional Specific Genetic Programs and a Role for cAMP in Lineage Specification.” *Cellular and molecular gastroenterology and hepatology* vol. 19,9 (2025): 101534. doi:10.1016/j.jcmgh.2025.101534
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