Benchmarking RepliGut® Planar-Jejunum intestinal epithelium against Caco-2 cells using RNAseq

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Introduction

The small intestine (SI) epithelium performs several functions that are critical for gastrointestinal health including:

- **Maintaining and renewing the physical barrier against intestinal contents**
- **Absorbing and metabolizing nutrients and medications**
- **Producing mucus to lubricate intestinal contents and prevent bacterial infections**
- **Communciating with the immune and nervous systems**
- **Facilitating the transport and elimination of bodily waste**

Studying intestinal processes in cell culture has often relied on colon tumor cells (Caco-2 or HT29) or immortalized murine cell lines, yet many properties of these cell lines are inconsistent with functions of normal SI enterocytes.[1–3] Caco-2 cells have been the workhorse of in vitro intestinal cell models, but possess uncontrolled proliferation properties, do not appropriately differentiate into mature enterocytes or mucus-secreting goblet cells, and have altered expression of drug-related transporters and enzymes compared to native human SI. Furthermore, Caco-2 cells are derived from the colorectal tumor of a single patient, precluding study of other intestinal regions or biological diversity across multiple donors.

Altis Biosystems has addressed these limitations by developing RepliGut® Systems, a series of intestinal stem cell (ISC)-derived cultures derived from organ transplant donors. ISCs plated onto a semi-permeable membrane differentiate into multiple cell lineages including enterocytes, goblet cells, and enteroendocrine cells **(Figure 1A)**. RepliGut® Systems maintain regional specificity and recapitulate aspects of the native human epithelium, such as intestinal barrier function, drug transport, metabolism, and mucus production. In contrast to Caco-2 cells which require 3 weeks in culture to mature, RepliGut® Systems reach maturity within 7-9 days, depending on the intestinal region **(Figure 1B)**. When jejunum progenitor cells are used, the RepliGut® Planar-Jejunum timeline consists of a cell proliferation phase (4-5 days) followed by a cell maturation phase (4-6 days) in which cells polarize and commit to absorptive and secretory cell lineages.

To highlight the critical differences between RepliGut® Planar-Jejunum and Caco-2 models, gene expression signatures were compared with a focus on several physiologic functions of the SI: (1) cell differentiation, (2) drug metabolism and transport, (3) cell-cell junction organization, and (4) intestinal absorption. Clear differences in gene expression were evident, which support the improved value of primary cell driven models like RepliGut® Systems for studying intestinal physiology in health and disease.

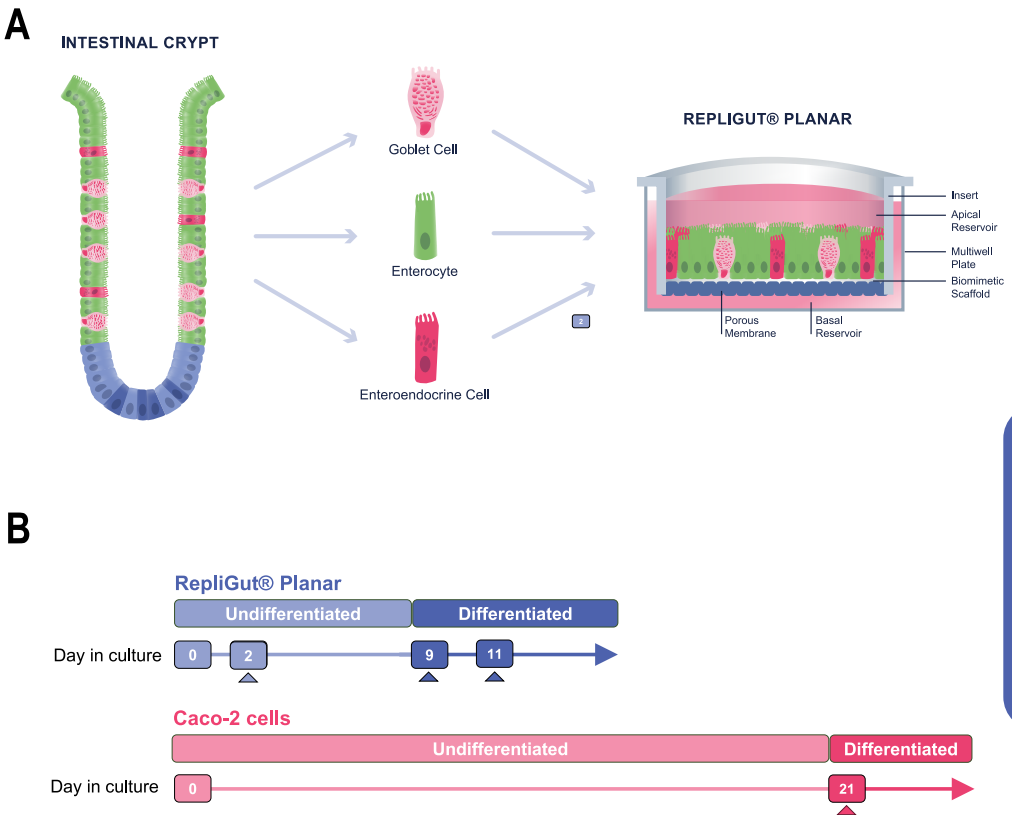

Figure 1 – (A) Schematic of RepliGut® Planar-Jejunum (B) Culture timelines of RepliGut® Planar-Jejunum and Caco-2 cells. Triangles represent the days of RNA collection. RNA was harvested from RepliGut® Planar-Jejunum in the undifferentiated phase (day 2), and in the mature phase (days 9 and 11).

Methods

CELL CULTURE

RepliGut® Planar– Jejunum cultures were established using the RepliGut® Planar - 96 Well - Small intestine – Jejunum Kit (RPG-96W-JEJ) as described using product instructions. Briefly, human ISCs from a single donor were plated on pre-coated semi-permeable membrane inserts in a 96-well plate to permit apical and basal access to cell monolayers. Cells were cultured in RepliGut® Growth Medium to allow expansion to confluence, then growth media was replaced by RepliGut® Maturation Medium to promote lineage specification to the absorptive and secretory fates. RNA was harvested in the growth phase before cells reached confluence (day 2 in culture), and in the mature phase on days 9 and 11 **(Figure 1B)**.

RNAseq LIBRARY PREPARATION AND SEQUENCING

mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. cDNA synthesis was performed NEBNext Ultra II RNA Library Prep kit for Illumina. Qubit, RT-PCR, and Bioanalyzer were used to determine cDNA quantity and size distribution. Libraries were pooled and sequenced on an Illumina platform to generate paired-end reads.

For publicly available gene expression data from Caco-2 cells, specific criteria were outlined to ensure the closest possible comparison to gene expression data from the RepliGut® Planar-Jejunum model: (1) Caco-2 cells must be cultured on a PET cell culture insert that allows access to apical and basal media compartments, (2) Caco-2 cells must undergo at least 18 days of spontaneous differentiation, (3) Caco-2 cells must be untreated (an experimental control group), and (4) Read count data must contain at least 20 million reads. Two publicly available RNAseq datasets from differentiated Caco-2 cells were identified to meet these criteria and obtained from the Gene Expression Omnibus (GSE226661 and GSE 223605).

RNAseq ANALYSIS

Standard RNASeq quality control and data processing steps were performed on all raw data. Fastq files were aligned to GRCh38 v.110 from Ensembl with Salmon v1.10.0 to generate transcript abundance counts. Differential gene expression analysis was performed with DESeq2 on raw count data.[4] A differentially expressed gene (DEG) was determined if there was q value <0.05, at least one Log_2 fold change difference between the cell types, and measurable expression in both sample groups.[5] For data visualization, gene expression values were obtained by Trimmed Mean of M-values (TMM) normalization across all samples using the Trinity (v2.15.1) and EdgeR (v4.0.16) packages, followed by log transformation and scaling to zero mean and unit variance.[6,7] TMM normalization assumes that the majority of genes, common to both samples, are not differentially expressed, and can reliably account for variation from different library sizes and widely different library compositions.

Results

RNAseq REVEALS REPLIGUT® PLANAR RECAPITULATES GENE EXPRESSION PATTERNS OF DIFFERENTIATED HUMAN INTESTINAL EPITHELIUM

RepliGut® Planar-Jejunum accurately mirrors the gene expression patterns found in the differentiated intestinal epithelium and deviates from the Caco-2 expression profile. DEseq2 identified 4,272 enriched genes in RepliGut® Planar-Jejunum over Caco-2 cells, and 4,135 enriched genes in Caco-2 cells over RepliGut® Planar-Jejunum. Principal component analysis highlights the separation between RepliGut® Planar-Jejunum and Caco-2 cells captured with the first principal component **(Figure 2,** 55.2% of gene expression variation). The second principal component captures 27.5% of the variation and mainly separates the growth-phase primary cells from the mature-phase primary and Caco-2 cells.

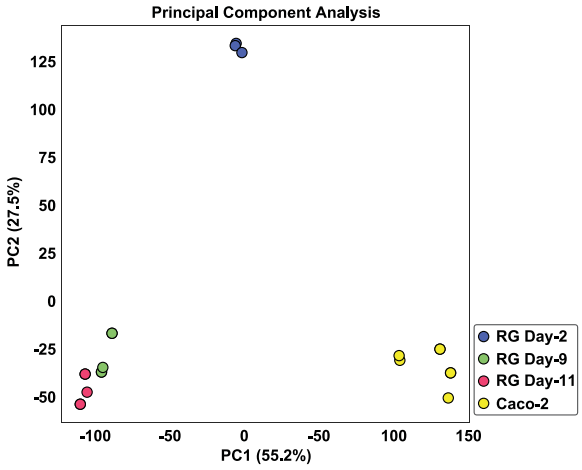

Figure 2 – Principal Component Analysis of RepliGut® Planar-Jejunum and Caco-2 RNAseq datasets. RG: RepliGut®; Day 2: growth phase; Day 9, Day 11: mature phase.

Several known markers of representative SI cell types were selected to illustrate the physiologic fidelity of the two culture models. As expected, known stem cell marker genes *LGR5* and *OLFM4* are highly expressed in the proliferative phase and minimally expressed in the mature phase of RepliGut® Planar-Jejunum **(Figure 3,** RG Day 9). As RepliGut® Planar-Jejunum progresses through the mature phase, high expression of intermediate enterocyte markers (*DMBT1, FABP1*) and differentiated enterocyte markers (*ALPI, MGAM*) are consistent with differentiation processes from previous reports and the native SI tissue.[9,10] The goblet cell marker, Mucin 2 (*MUC2*), also increases in expression throughout the RepliGut® Planar-Jejunum culture timeline. RepliGut® Planar-Jejunum is significantly enriched for 11 of 17 mucin-coding genes, with the remaining six genes below minimum expression thresholds for the entire dataset (data not shown).

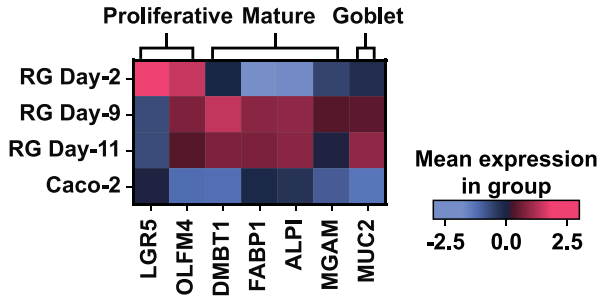

Figure 3 – Cell lineage markers expressed in RepliGut® Planar-Jejunum and differentiated Caco-2 cells. Blue represents low expression. Red represents high expression. RG: RepliGut®; Day 2: growth phase; Day 9, Day 11: mature phase.

Caco-2 cells show conflicting expression of these cell type markers. Simultaneous expression of proliferative cell marker *LGR5* with enterocyte markers *FABP1* and *ALPI* suggests that Caco-2 cell gene expression is inconsistent with mature human SI enterocytes. Furthermore, a lack of expression of *MGAM* and *MUC2* supports the well-established assertion that Caco-2 cells do not recapitulate the complexity of mixed cell subtypes of the native SI.

GENE EXPRESSION COMPARISON OF INTESTINAL PHYSIOLOGIC PROCESSES

Gene Ontology (GO) enrichment analysis revealed RepliGut® Planar- Jejunum is enriched for expression of several critical SI physiologic processes as compared to Caco-2 cells. Within the GO gene terms xenobiotic metabolic process, cell-cell junction organization, and intestinal absorption, 53 of 125 genes (42.4%), 71 of 179 genes (39.7%) and 20 of 32 genes (62.5%) are enriched in RepliGut® Planar- Jejunum, respectively **(Figure 4)**. We further explored these gene enrichments in alignment with know key mechanistic processes.

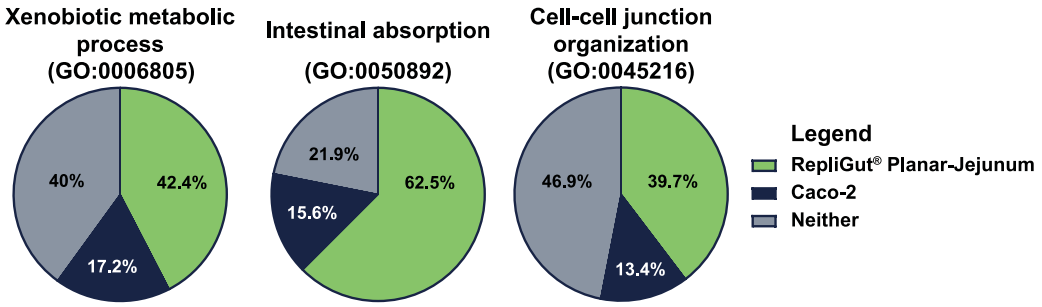

Figure 4 – Pie charts showing breakdown of gene expression enrichment in RepliGut® Planar-Jejunum, Caco-2, or neither from selected GO terms.

ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION

Orally administered therapeutics must pass through the single-cell thick intestinal epithelium before they can enter systemic circulation. In vitro modeling of this process requires the presence and functionality of the appropriate influx and efflux transporters as well as metabolic enzymes. RepliGut® Planar-Jejunum is enriched for several genes involved in intracellular transport. Genes encoding P-gp (*ABCB1*; *MDR1*), BCRP (*ABCG2*), and MRP1 (*ABCC1*) each have at least a 2-fold enrichment in RepliGut® Planar-Jejunum compared to Caco-2 cells **(Figure 5)**. Additionally, RepliGut® Planar-Jejunum demonstrates significant enrichment for genes from each of the major classes of drug-metabolizing enzymes (i.e., Cytochrome P450s, UGTs, SULTS, and CESs). CYP3A4, the most prevalent phase 1 metabolism enzyme in the liver, expression was significantly enriched compared to Caco-2 cells. Caco-2 cells are enriched for the carboxylesterase-encoding gene *CES1*, which is not representative of native SI.[11] RepliGut® Planar-Jejunum gene expression signatures accurately reflect the native SI with low *CES1* expression and high *CES2* expression. These enrichment patterns support a critical role for the SI in first pass metabolism and highlight the shortcomings of using Caco-2 cells in preclinical testing of orally administered medications.[1,12]

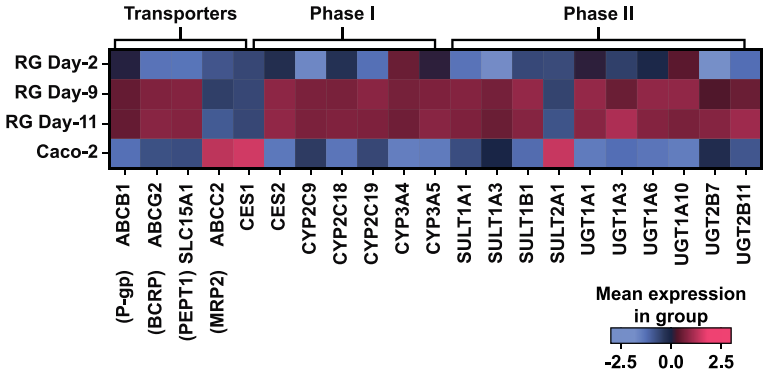

Figure 5 - RepliGut® Planar-Jejunum shows significantly higher expression of critical transporters, Phase I, and Phase II metabolism genes compared to Caco-2 cells. Blue represents low expression. Red represents high expression. RG: RepliGut®; Day 2: growth phase; Day 9, Day 11: mature phase.

EPITHELIAL BARRIER FUNCTION

Cell-cell junctions regulate the permeability of the intestinal barrier, selectively allowing nutrient absorption while blocking the entry of pathogens and toxins. Although Caco-2 cells can form a functional barrier, the composition of the junctions binding these cells is markedly different than those found in the native SI.[1] Genes encoding for important cell-cell junctions such as e-Cadherin (*CDH1*), ZO-1 (*TJP-1*), and multiple Claudins are all significantly enriched in RepliGut® Planar-Jejunum **(Figure 6)**.

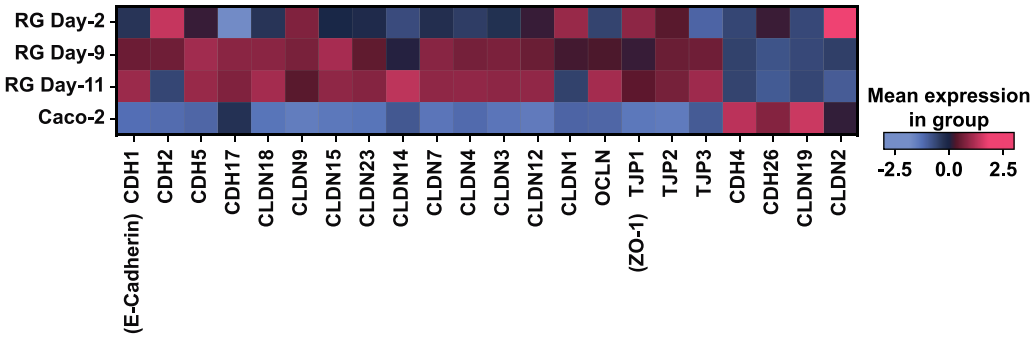

Figure 6 - Enrichment of genes within the cell-cell junction organization GO Biological Process term (GO: 0045216). Blue represents low expression. Red represents high expression. RG: RepliGut®; Day 2: growth phase; Day 9, Day 11: mature phase.

INTESTINAL NUTRIENT ABSORPTION

Another major function of the intestine is to absorb and metabolize food into energy and nutrients for the entire body. Nutrient absorption pathways for all major biomolecules are enriched in RepliGut® Planar-Jejunum for key genes that mediate dietary nutrient uptake and metabolism. *FABP1*, *FABP2*, and *CD36* bind long-chain fatty acids and are important for uptake of long chain fatty acids in the SI **(Figure 7)**. *NPC1L1*, *ABCG5*, and *ABCG8* are involved with cholesterol uptake and excretion.[13] *SLC5A1* (SGLT1) is the primary mediator of dietary glucose and galactose uptake and *ALDOA*, *ALDOB*, and *ALDOC* are aldolases that function in glycolysis and gluconeogenesis. *MGAM* and *SI* synergize to digest dietary starches while *LCT*, or lactase, breaks lactose into glucose and galactose.[13] *SLC15A1* (PEPT1), *SLC6A14*, and *SLC6A19* each mediates uptake of different classes of dietary proteins and peptides.[13] Altogether, the comprehensive enrichment for biomolecule metabolism and absorption in RepliGut® Planar-Jejunum is consistent with expression patterns in native tissues where the SI, specifically the jejunum, is the primary site of dietary absorption and metabolism.

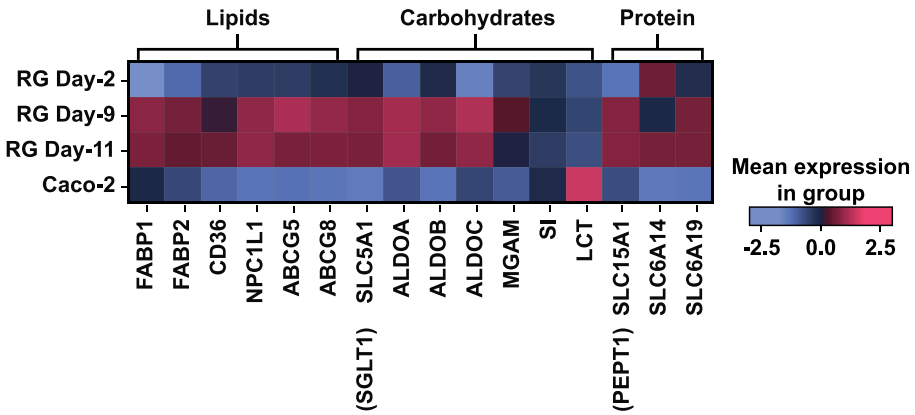

Figure 7 - Enrichment of genes for dietary lipid, carbohydrate, and protein absorption and metabolism in RepliGut® cultures over Caco-2 cells. Blue represents low expression. Red represents high expression. RG: RepliGut®; Day 2: growth phase; Day 9, Day 11: mature phase.

Conclusion

When compared to Caco-2 cells, which are one of the most widely used pre-clinical models of intestinal physiology, RepliGut® Planar-Jejunum demonstrates an overall closer representation of normal intestinal physiology and distinguishes itself from conventional Caco-2 cells through a host of critical physiological processes. RepliGut® Planar-Jejunum cultures demonstrate superior fidelity to Caco-2 cells, exhibiting enrichment for genes involved in crucial functions such as cell differentiation, drug metabolism and transport, cell-cell junction organization, and nutrient absorption. These data highlight the necessity for using primary intestinal cultures for studying any major enteric physiologic processes.

References: 1. Fedi, A. et al. In vitro models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review. *J. Controlled Release* 335, 247–268 (2021). **2.** O'Shea, J. P. et al. Best practices in current models mimicking drug permeability in the gastrointestinal tract - An UNGAP review. *Eur. J. Pharm. Sci.* 170, 106098 (2022). **3.** Markus, J. et al. Human small intestinal organotypic culture model for drug permeation, inflammation, and toxicity assays. *Vitro Cell. Dev. Biol.* - Anim. 57, 160–173 (2021). **4.** Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014). **5.** Zhao, Y. et al. TPM, FPKM, or Normalized Counts? A Comparative Study of Quantification Measures for the Analysis of RNA-seq Data from the NCI Patient-Derived Models Repository. *J. Transl. Med.* 19, 269 (2021). **6.** Haas, B. J. et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8, 1494–1512 (2013). **7.** Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140 (2010). **8.** Takayama, K. et al. In Vivo Gene Expression Profile of Human Intestinal Epithelial Cells: From the Viewpoint of Drug Metabolism and Pharmacokinetics. *Drug Metab. Dispos.* 49, 221–232 (2021). **9.** Gomez-Martinez, I. et al. A Planar Culture Model of Human Absorptive Enterocytes Reveals Metformin Increases Fatty Acid Oxidation and Export. *Cell. Mol. Gastroenterol. Hepatol.* 14, 409–434 (2022). **10.** Burclaff, J. et al. A Proximal-to-Distal Survey of Healthy Adult Human Small Intestine and Colon Epithelium by Single-Cell Transcriptomics. *Cell. Mol. Gastroenterol. Hepatol.* 13, 1554–1589 (2022). **11.** Taketani, M., Shii, M., Ohura, K., Ninomiya, S. & Imai, T. Carboxylesterase in the liver and small intestine of experimental animals and human. *Life Sci.* 81, 924–932 (2007). **12.** Sharma, A., Jin, L., Wang, X., Wang, Y.-T. & Stresser, D. M. Developing an adult stem cell derived microphysiological intestinal system for predicting oral prodrug bioconversion and permeability in humans. *Lab. Chip* (2023) doi:10.1039/D3LC00843F. **13.** Kiela, P. R. & Ghishan, F. K. Physiology of Intestinal Absorption and Secretion. *Best Pract. Res. Clin. Gastroenterol.* 30, 145–159 (2016).

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ABOUT ALTIS BIOSYSTEMS, INC.

Altis Biosystems developed a series of in vitro, stem cell derived intestinal cell models called Repligut® Systems for use in pre-clinical drug discovery. RepliGut® Systems address current limitations of cell lines and animal models for the accurate representation of human intestinal physiology. Our flagship RepliGut® Planar models support donor intestinal stem cells each isolated from different regions of the intestine that are sequentially proliferated and differentiated into the key cell types in the human gastrointestinal epithelium. RepliGut® Models developed from the different intestine regions recreate the cellular lifecycle of the intestinal crypt and can be used for compound screening, disease modeling, drug absorption, toxicity, efficacy, and basic research studies. We are proud to have industry leading scientists on our team that share a passion for creating high-throughput, scalable, and translatable products and services to support accelerated drug development.

PRODUCTS AND SERVICES

RepliGut® Systems are derived from stem cells from individual regions of the gut, each of which are already committed in fate to a certain section of the gut. Therefore offering a more precise query of the gut that can't be accomplished using tumor-derived or iPSC cell models.

KITS

Establish RepliGut® Models from 5 different intestinal regions in your lab using quality-controlled reagents and protocols combined together into a kit. Select from multiple donor demographics to suit your needs.

SKU	Product Type	Product Name
RGP-12W-DUO RGP-12W-JEJ RGP-12W-TC RGP-12W-AC RGP-12W-DC	RepliGut® Kit 12-well formats for all regions	RepliGut® Planar – 12 Well – Small Intestine – Duodenum RepliGut® Planar – 12 Well – Small Intestine – Jejunum RepliGut® Planar – 12 Well – Transverse Colon RepliGut® Planar – 12 Well – Ascending Colon RepliGut® Planar – 12 Well – Descending Colon
RGP-96W-DUO RGP-96W-JEJ RGP-96W-TC RGP-96W-AC RGP-96W-DC RGP-96W-JEJ-T RGP-96W-TC-T	RepliGut® Kit 96-well formats for all regions	RepliGut® Planar – 96 Well – Small Intestine – Duodenum RepliGut® Planar – 96 Well – Small Intestine – Jejunum RepliGut® Planar – 96 Well – Transverse Colon RepliGut® Planar – 96 Well – Ascending Colon RepliGut® Planar – 96 Well – Descending Colon RepliGut® Planar – 96 Well – Small Intestine – Jejunum – Trial Kit RepliGut® Planar – 96 Well – Transverse Colon – Trial Kit

CUSTOM SERVICES

Let our expert researchers deliver the data using a variety of established assays using RepliGut® Planar models. Assay not listed? Ask us for more information on assay development. Contact scientificsupport@altisbiosystems.com

Cell Monitoring	Bioassays	Transport	Protein	RNA Isolation and cDNA generation	Gene Expression	Staining/ Imaging
Bright Field Microscopy TEER	Cytotoxicity Permeability Cell Titer Glo	LC/MS	ELISA Luminex	RNA Isolation RNA Quantitation RNA Quality cDNA generation	BioMark QuantiGene	Cell proliferation Antibody-based staining Imaging