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APPLICATION NOTE

RNA-SEQ ANALYSIS VALIDATES THE SUITABILITY OF PEPTONE-FREE MCCOY'S 5A MEDIA FOR CELL CULTURE

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ABSTRACT

In our previous study, we demonstrated that the newly developed McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) can serve as a viable substitute for the existing McCoy's 5A Medium (ATCC® 30-2007™) in cell culture applications as there was no significant impact to cell health or proliferation for the cell lines evaluated. However, changes in medium formulations run the risk for possible shifts in gene expression. In the following study, we evaluated the gene expression profiles of U-2 OS and HCT 116 cells grown in the different medium formulations. Whole-transcriptome profiling (RNA-seq) showed only a small number of genes were differentially expressed in cells grown in the peptone-free medium, and none of these genes are functionally involved in regulating cell proliferation or viability. Bioinformatics analysis further confirmed that the differentially expressed genes were not associated with pathways related to cell growth and viability, reinforcing the suitability of McCoy's 5A Medium, Peptone-free as a replacement for the standard medium in cell culture applications.

INTRODUCTION

Successful cell culture depends on using media that deliver the necessary nutrients, growth factors, and environment for cells to thrive. Traditionally, animal by-products (ABPs) have been integral components of cell culture media formulations. For instance, the widely used McCoy's 5A Medium formulation has evolved over time to include animal-derived peptones to supply an additional nitrogen source. However, concerns have arisen regarding the potential introduction of adventitious pathogens through ABPs,[1,2] posing a significant safety risk when culturing cells to produce biologics, vaccines, and other products with therapeutic applications. This risk has prompted the development of ABP-free alternatives, such as our newly developed McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™).

In our previous study,[3] we evaluated the performance of our peptone-free medium against the standard McCoy's 5A medium formulation and found it to be a suitable substitute that imparts no significant impact to cell health or proliferation. The novel medium not only successfully supported the long-term growth of cell lines currently cultured in the original McCoy's 5A medium but also exerted no cytotoxic effects on any cell line tested. To further validate the use of McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) as an alternative to the standard formulation, we sought to evaluate the transcriptomic landscape of cell lines cultured in the medium.

When culturing cells, the environment can influence gene expression and phenotype. While environmental changes can trigger natural developmental processes, they can also impart harmful effects that impact development and disease.[4,5] Various environmental factors such as nutrition, chemical exposure, temperature fluctuations, and other stresses can alter phenotypes and regulate gene expression epigenetically in experimental model systems.[6-8] Epigenetic mechanisms in cells are driven by DNA methylation and stable chromatin

modifications.[9] There is growing interest in understanding how environmental factors influence the establishment and maintenance of epigenetic modifications, which, in turn, affect gene expression and phenotype.[10] Nutrients are recognized as epigenetic factors, and changes in media formulations can lead to shifts in gene expression.[1,2,11] Therefore, understanding gene expression patterns is critical for assessing the suitability of media for sustainable cell culture and research purposes.

In this study, we assessed the transcriptomic profiles of the tumor cell lines HCT 116 (ATCC® CCL-247™) and U-2 OS (ATCC® HTB-96™) following growth in the McCoy's 5A Medium, Peptone-free (ATCC® 30-2011™) and compared them to that of cells grown in the standard McCoy's 5A Medium (ATCC® 30-2007™). Our analysis included a comparison of gene expression patterns, Ingenuity Pathway Analysis (IPA) and gene ontology analysis of the differentially expressed genes, and relative mRNA levels were evaluated. The findings demonstrate that the gene expression profiles of cells cultured in the peptone-free medium are comparable to those of cells grown in the standard medium, with minimal impact on key biological pathways. These results strongly support the use of the new McCoy's medium as an effective alternative to the standard formulation for cell culture.

MATERIALS & METHODS

CELL LINES:

In this study, we evaluated the performance of McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) using the following cell lines: HCT 116 (ATCC® CCL-247™) and U-2 OS (ATCC® HTB-96™). The cell lines selected utilize the current ATCC McCoy's 5A medium as their basal medium.

PREPARATION OF COMPLETE CELL CULTURE MEDIA FOR TESTING:

Complete culture media for each cell line was prepared in accordance with ATCC cell line specifications. Control medium was prepared using McCoy's 5A Medium (ATCC® 30-2007™) as the basal media. Test medium was prepared using McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) as the basal media. All preparations used the same lot of fetal bovine serum (FBS; ATCC® 30-2020™) to control for variations in sera lots. Cells were thawed directly into complete culture media containing McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) without an adaption period for all experiments.

RNA EXTRACTION AND QUALITY CONTROL:

RNA isolation was performed using the QIAGEN® QIAcube® automated system with the RNeasy® Mini QIAcube® Kit. Frozen samples were thawed and prepared for RNA extraction according to ATCC's work instructions. Extracted samples were tested for RNA integrity and quality using the Agilent® TapeStation® (RNA Integrity Number (RIN) ≥ 6.5), RNA purity using the Thermo Fisher Scientific® Nanodrop® (A260/A280 1.8 ≥ x ≤ 2.2), and concentration using the Qubit®.

RNA-SEQ LIBRARY PREPARATION AND SEQUENCING:

Automated RNA-seq next-generation sequencing (NGS) library preparation was performed on an Eppendorf® epMotion® 5075 Liquid Handler using the Illumina® Stranded mRNA Prep, Ligation kit. Prepared NGS libraries were assessed using Invitrogen® Qubit® dsDNA High Sensitivity Assay Kit and an Agilent® 4200 TapeStation® and D5000 ScreenTape® System. Libraries were prepared using Illumina® P3 200-cycle Reagent kit and sequenced on the NextSeq® 2000 platform.

RNA-SEQ DATA ANALYSIS:

Our data analysis pipeline included quality control, read trimming, alignment to the reference transcriptome, and quantification of gene expression. Utilizing the CLC Genomics Workbench v23 (QIAGEN® Digital Insights), an end-to-end pipeline was created that, briefly, entailed the following steps. First, raw paired end Illumina® reads were trimmed and filtered to a minimum quality of Q30 and a maximum of 2 ambiguous bases. Furthermore, potential "read-through" adapter sequences and 3' polyG sequences (due to using the 2-color NextSeq® platform) were also automatically identified and trimmed. Reads below 50 bp were then discarded. Next, reads were mapped to the human genome hg38 reference genome (obtained from Ensemble) using the default settings for bulk-RNA-seq experiments: mismatch cost 2; InDel cost 3; length fraction 0.8; similarity fraction 0.8; maximum hits per reads 10; reversed strand-specific mapping 1; ignore broken read pairs 1. A minimum of 18M mapped reads per library were required for each biological replicate, and TMM normalization was carried out for each library.[12] Statistical comparisons between groups were conducted using a Wald test, and fold-change values calculated from the GLM model.[13] Outliers were down weighted and iteratively re-fit to the GLM model.[14] Low expression genes were filtered prior to FDR correction and calculation.[15] The gene list obtained from the differential gene expression analysis was further analyzed using the Ingenuity Pathway Analysis (IPA) platform. IPA automatically carried out statistical comparisons for gene-set enrichment analysis, statistically significant pathway activation, identification of upstream regulators and downstream targets, gene and pathway associated diseases and biofunctions. IPA was also used to produce network diagrams of pathways and associated differentially expressed genes.

BIOINFORMATICS ANALYSIS:

The gene list obtained from the differential gene expression analysis underwent Ingenuity Pathway Analysis (IPA). IPA generated comprehensive reports on the top canonical pathways, upstream regulators, diseases and biofunctions, regulator effect networks, gene interaction networks, tox lists, machine learning disease pathways, and analysis-ready molecules.

RESULTS

To assess the impact of culture medium formulation on the whole-genome transcriptomic landscape of cells, we conducted parallel cultures of U-2 OS (ATCC® HTB-96™) and HCT 116 (ATCC® CCL-247™) cell lines in both ATCC's newly developed McCoy's 5A Medium, Peptone-free (ATCC® 30-2011™) and the standard McCoy's 5A medium (ATCC® 30-2007™). After culture, cells were collected and processed to evaluate how the media formulations influenced inherent molecular characteristics and basal gene expression levels. RNA was extracted from each biological replicate and used for RNA sequencing (RNA-seq) to elucidate the transcriptomic landscape of cell lines cultured in the newly modified McCoy's 5A Medium, Peptone-free as compared to standard formulation (Figure 1).

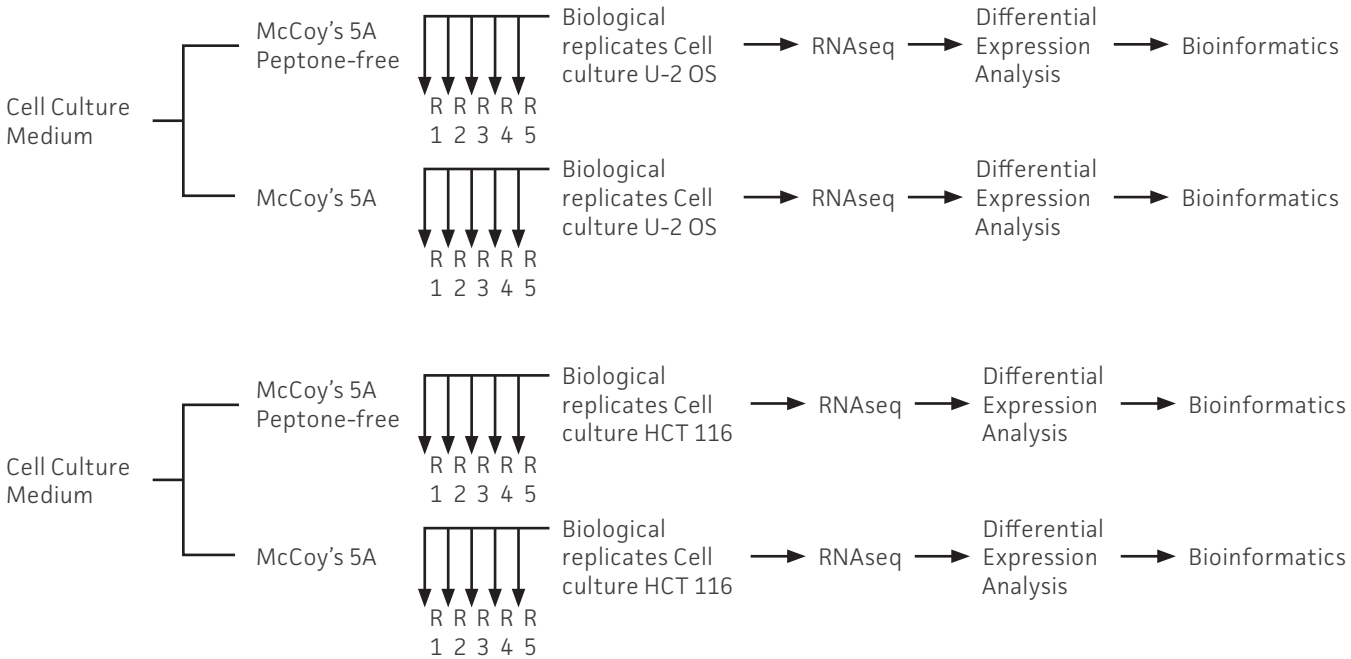


Figure 1: Experimental layout demonstrating cell culture, RNA sequencing, differential gene expression analysis, and bioinformatics analysis workflows. The workflows for U-2 OS are represented in the top two rows, and the workflows for HCT 116 are represented in the bottom two rows. R1, R2, R3, R4, and R5 indicate biological replicates of the cell culture.

GENE EXPRESSION PROFILE ANALYSIS OF U-2 OS CELLS

Principal component analysis (PCA) of U-2 OS cells revealed tight clustering of biological replicates within the same culture condition, while samples from different culture conditions formed two distinct clusters and slightly diverged from each other, indicating some changes in transcript and gene expression levels in U-2 OS cells. This suggests that distinct culture media may produce phenotypic differences in U-2 OS cells in response to the change in medium formulation (Figure 2A).

Next, we conducted comparative gene expression analysis between the samples, which identified differentially expressed genes (DEGs), providing insights into how medium formulation influences gene expression. Specifically, our differential gene expression analysis revealed genes that were either upregulated or downregulated in cells grown in the new McCoy's 5A Medium, Peptone-free (ATCC® 30-2011™) relative to the standard formulation. Interestingly, only 54 out of 47,467 genes—including coding and non-coding—were altered in U-2 OS (ATCC® HTB-96™) cells cultured in the new peptone-free medium (Figure 2B), indicating a minimal shift in gene expression despite the change in medium formulation (Figure 2C).

Further analysis identified just three DEGs (FOS, AHRR, CCKAR) enriched at a minimal basal level (with a threshold cutoff value of 10 reads per transcript) associated with the new peptone-free medium, further supporting its suitability as an alternative to the standard medium for U-2 OS cell culture (Figure 2C, 2D).

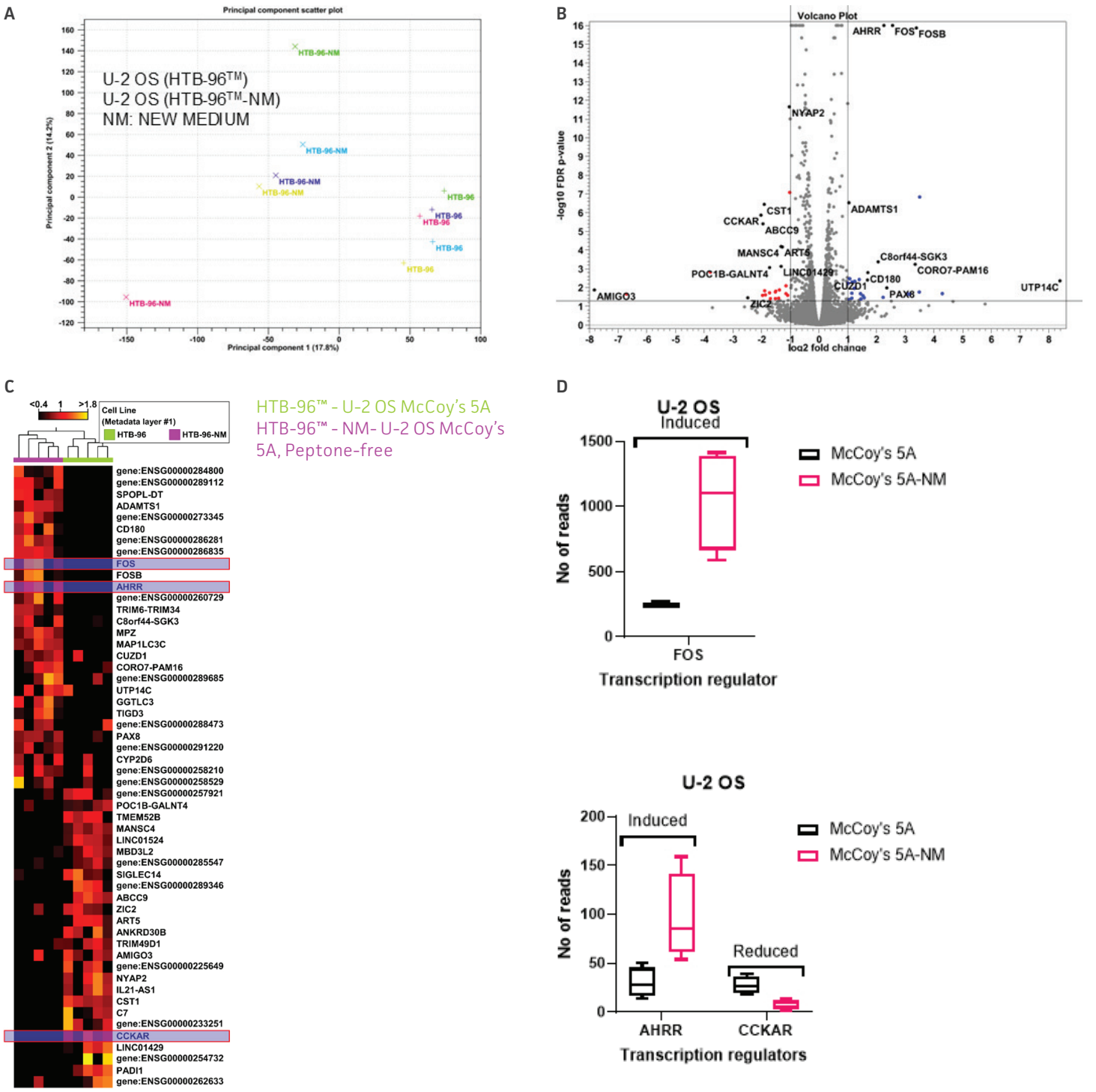


Figure 2: Transcriptional landscape of U-2 OS (ATCC® HTB-96™) cells cultured in McCoy's 5A Medium, Peptone-free versus the standard formulation. (A) Principal Component Analysis (PCA) of U-2 OS samples cultured in the new peptone-free versus standard McCoy's 5A medium formulation. Samples are grouped based on gene expression variance. (B) Expression pattern of all genes in U-2 OS cells cultured in the new McCoy's 5A Medium, Peptone-free as compared to the standard McCoy's 5A Medium formulation. A volcano plot displays the pattern of differentially expressed genes (DEGs) in U-2 OS cells. The plot includes all genes, highlighting those considered DEGs by using thresholds for both adjusted p-value (0.05, y-axis) and fold-change (2, x-axis). Dark gray indicates non-significant genes (both fold-change and adjusted p-value fall below the thresholds), while blue (upregulated) and red (downregulated) dots represent significant DEGs. (C) Expression patterns of DEGs in U-2 OS cells cultured in McCoy's 5A Medium, Peptone-free versus standard McCoy's 5A Medium. A heatmap illustrates the expression patterns of DEGs, with combined clustering methods grouping genes and/or samples based on the similarity of their expression profiles. Rows represent genes, while columns represent samples. The color and intensity of the boxes depict relative changes (not absolute values) in gene expression. In the heatmap, red represents upregulated genes while black represents downregulated genes. (D) Box plots display the relative basal mRNA levels of DEGs in U-2 OS cells cultured in McCoy's 5A Medium, Peptone-free. NM: New Medium.

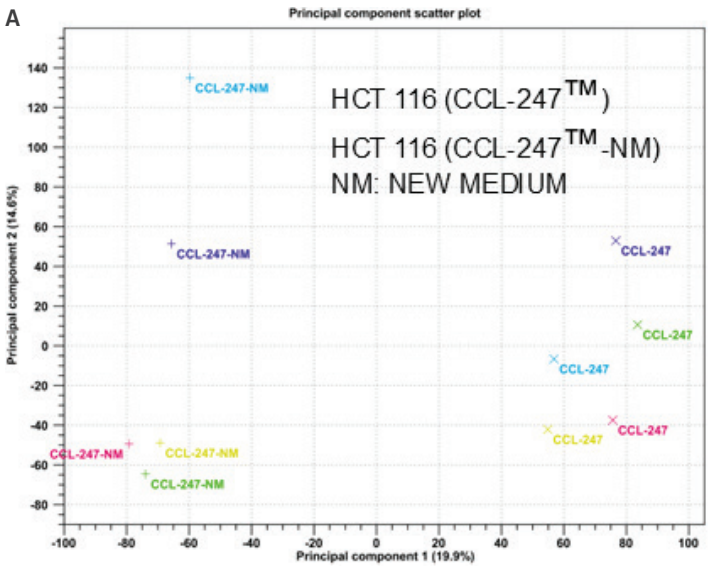
GENE EXPRESSION PROFILE ANALYSIS OF HCT 116 CELLS

To validate the robustness of the new McCoy's 5A Medium, Peptone-Free (ATCC® 30-2011™) across other cell types, we extended our analysis to the widely used HCT 116 cell line (ATCC® CCL-247™). Again, PCA showed close clustering of biological replicates within a specific culture condition, while samples grown in different media conditions clustered separately (Figure 3A). This analysis revealed a clear distinction between the two groups that was likely driven by variations in gene expression between HCT 116 cells cultured in the new peptone-free medium and those in the standard medium.

To determine the gene expression variance in the HCT 116 cells cultured in McCoy's 5A Medium, Peptone-free as compared to the standard medium, we then performed differential expression analysis on the HCT 116 cells. This analysis revealed that out of the 47,467 genes analyzed—including both coding and non-coding genes—only 124 genes were differentially expressed in cells grown in McCoy's 5A Medium, Peptone-free as compared to those grown in standard McCoy's 5A Medium (Figure 3B). Further examination of basal enrichment, using a threshold of 10 reads per transcript, uncovered that 66 of the 124 DEGs were enriched in HCT 116 cells under both medium conditions (Figure 3C, 3D). This suggests that McCoy's 5A Medium, Peptone-free may have introduced a certain degree of transcriptional variability in HCT 116 cells.

IPA revealed several dysregulated pathways. Among the top ten, only two exhibited significant changes: the EIF2AK1 (HRI) pathway was activated while the Xenobiotic Metabolism AHR Signaling Pathway was deactivated (Figure 3E). There was no activity observed in the other dysregulated pathways. We then conducted gene ontology analysis on the 66 enriched DEGs and visualized their expression patterns through heatmaps following growth of HCT 116 cells in McCoy's 5A Medium, Peptone-free versus the standard McCoy's 5A Media. This analysis revealed that the DEGs were involved in transcription regulation, kinase activity, transport, and enzymatic processes (Figure 3F, 3G, 3H, 3I). Subsequent quantitative analyses show the relative basal level of the filtered DEGs with the threshold cutoff values (Figure 3J, 3K, 3L).

A



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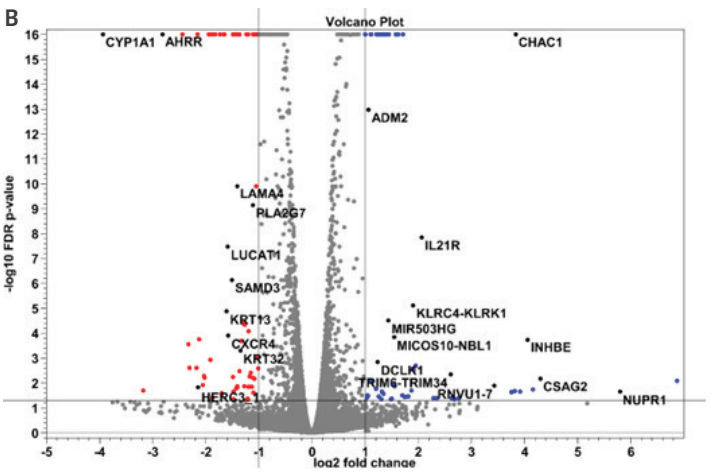

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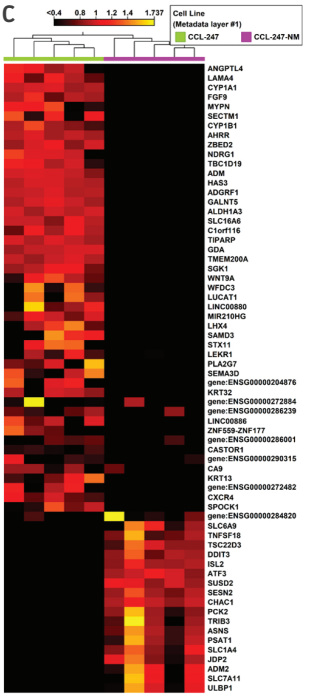
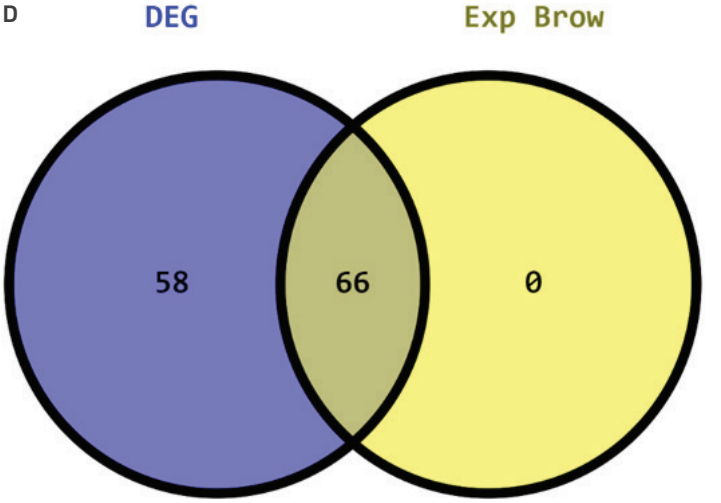


CCL-247™ - HCT 116 McCoy's 5A
CCL-247™-NM - HCT 116 McCoy's 5A peptone-free; NM: NEW MEDIUM

D



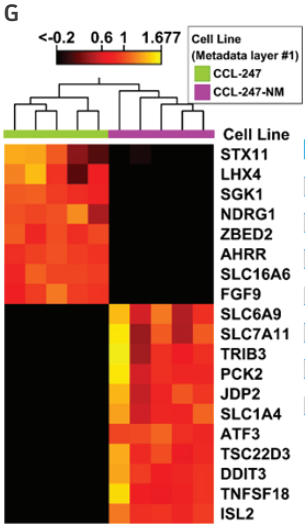
E

Ingenuity Canonical Pathways	-log(p-)	Ratio	z-score	Molecules
Response of EIF2AK1 (HRI) to heme deficiency	9.79	0.33	2.24	ASNS, ATF3, CHA C1, DDIT3, TRIB3
Xenobiotic Metabolism AHR Signaling Pathway	4.31	0.05	-1	AHRR, ALDH1A3, CYP1A1, CYP1B1
Response of EIF 2AK4 (GCN2) to amino acid deficiency.	4.02	0.04	2	ASNS, ATF3, DDIT3, TRIB3

F

Name	Fold	p-value	Molecular Function
TSC22D3	3.084	4.4E-50	transcription regulator
DDIT3	3.048	4.09E-70	transcription regulator
JDP2	2.739	2.12E-49	transcription regulator
ATF3	2.334	8.16E-75	transcription regulator
ISL2	2.147	3.4E-18	transcription requator
LHX4	-2.399	0.0000475	transcription regulator
ZBED2	-2.796	5.06E-84	transcription regulator
AHRR	-7.018	6.28E-150	transcription regulator
PCK2	2.632	2.55E-57	kinase
TRIB3	2.171	3.88E-47	kinase
NDRG1	-2.061	1.49E-75	kinase
SGK1	-2.3	2.06E-18	kinase
FGF9	-3.835	2.96E-39	growth factor
SLCTA11	2.479	1.85E-43	transporter
SLC1A4	2.411	3.48E-28	transporter
SL C6A9	2.408	2.48E-18	transporter
STX 11	-2.045	0.00101	transporter
SLC16A6	-3.319	1.72E-68	transporter

G



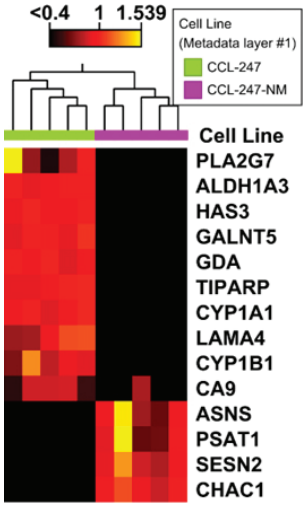
CCL-247™ - HCT 116 McCoy's 5A

CCL-247™-NM - HCT 116 McCoy's 5A, peptone-free; NM: NEW MEDIUM

H

Name	Fold	p-value	Function
CYP1A1	-15.275	1.43E-93	enzyme
GALNT5	-4.457	2.6E-270	enzyme
ALDH1A3	-3.665	2.2E-277	enzyme
HAS3	-3.536	5.5E-244	enzyme
TIPARP	-3.148	1.6E-181	enzyme
CYP1B1	-3.132	1.63E-19	enzyme
LAMA4	-2.644	1.26E-10	enzyme
GDA	-2.345	2.8E-107	enzyme
CA9	-2.194	0.0141	enzyme
PLA2G7	-2.153	7.2E-10	enzyme
PSAT1	2.012	1.35E-66	enzyme
ASNS	2.572	6.53E-93	enzyme
SESN2	3.271	3.8E-132	enzyme
CHAC1	14.3	0	enzyme

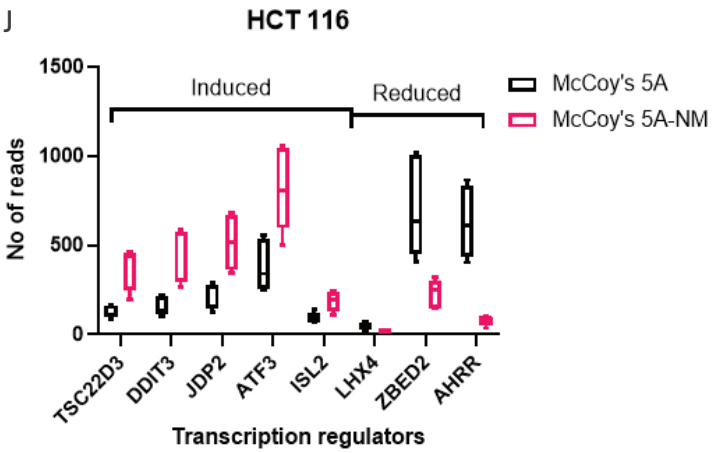
I

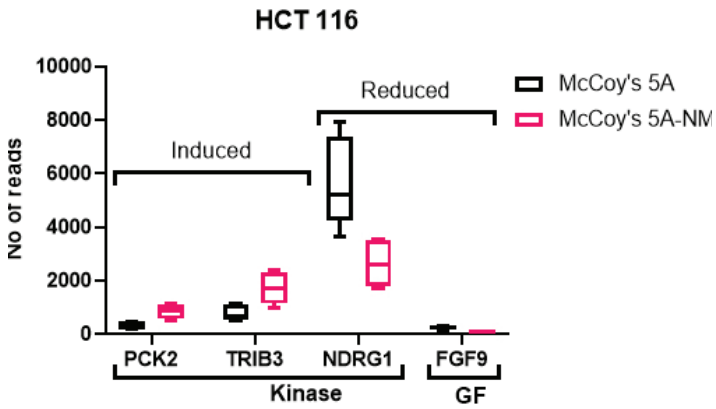


CCL-247™ - HCT 116 McCoy's 5A

CCL-247™-NM - HCT 116 McCoy's 5A, Peptone-free; NM: NEW MEDIUM

J





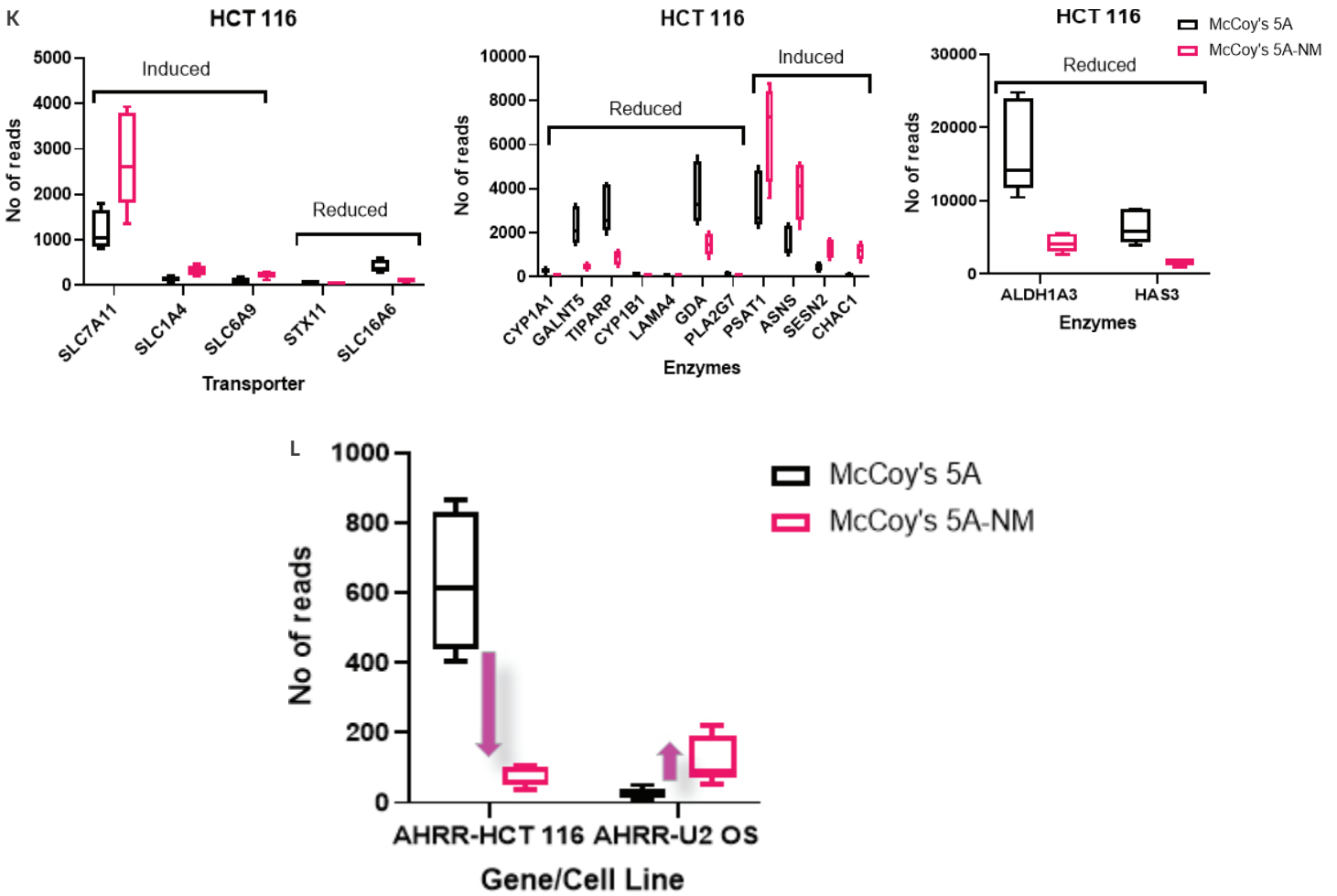


Figure 3: Transcriptional landscape of HCT 116 (ATCC® CCL-247™) cells cultured in McCoy's 5A Medium, Peptone-free versus the standard formulation. (A) Principal Component Analysis (PCA) of HCT 116 samples cultured in peptone-free versus conventional McCoy's 5A medium, with samples grouped based on gene expression variance. (B) Expression patterns of all genes in HCT 116 cells cultured in the McCoy's 5A Medium, Peptone-free as compared to the standard formulation. A volcano plot illustrates the pattern of differentially expressed genes (DEGs) in HCT 116 cells. The plot includes all genes, with DEGs highlighted using thresholds for both adjusted p-value (0.05, y-axis) and fold-change (2, x-axis). Dark gray indicates non-significant genes (both fold-change and adjusted p-value below the thresholds), while blue (upregulated) and red (downregulated) dots represent significant DEGs. (C) Expression patterns of DEGs in HCT 116 cells cultured in McCoy's 5A Medium, Peptone-free versus the standard McCoy's 5A Medium. A heatmap displays the expression patterns of DEGs, with clustering methods grouping genes and/or samples based on the similarity of their expression profiles. Rows represent genes, and columns represent samples. The color intensity represents changes (not absolute values) in gene expression, with red indicating upregulated genes and black indicating downregulated genes. (D) Venn diagram showing the total number of DEGs and those enriched at a basal threshold cutoff value of 10 reads per transcript. (E) A list of the top ten dysregulated Ingenuity Canonical Pathways along with their z-scores indicating activation status. (F) A list of differentially expressed transcription regulators, kinases, growth factors, and transporters that are enriched at a minimum basal cutoff level in HCT 116 cells. (G) A heatmap exhibiting the expression pattern of enriched DEGs shown in panel F. (H) A list of differentially expressed genes that function as enzymes that are enriched at a basal cutoff level in HCT 116 cells cultured in McCoy's 5A Medium, Peptone-free. (I) A heatmap showing the expression pattern of the differentially expressed enzymes enriched in HCT 116 cells. (J) Box plots showing the relative basal mRNA levels of transcription regulators, kinases, and growth factors in HCT 116 cells cultured in McCoy's 5A Medium, Peptone-free. (K) Box plots displaying the relative basal mRNA levels of genes functioning as transporters and enzymes in HCT 116 cells cultured in the McCoy's 5A medium peptone-free. (L) Box plot showing the relative mRNA abundance of the AHRR gene in U-2 OS and HCT 116 cells cultured either in the new McCoy's medium peptone-free or the standard formulation. NM: New Medium.

CONCLUSION

In this study, we examined effects of the recently produced McCoy's 5A Medium, Peptone-free (ATCC® 30-2011™) and existing McCoy's 5A Medium (ATCC® 30-2007™) on the transcriptome profiles of the U-2 OS and HCT 116 cell lines. Our investigation revealed that the peptone-free medium caused very little variations in the expression of genes as compared to the standard medium. To be more precise, only 54 of 47,467 genes in U-2 OS cells showed differential expression, suggesting that the transcriptome landscape of cells grown in the peptone-free formulation is like that of the conventional medium. A marginally greater number of genes (124) were differently expressed in HCT 116 cells, yet only 66 of them were enriched, indicating a limited influence on the overall profile of gene expression.

PCA revealed distinct clustering based on the culture conditions, highlighting potential phenotypic differences resulting from the medium formulation. Although the new peptone-free medium showed a slight transcriptional variability in HCT 116 cells, it did not significantly alter the signaling pathways associated with cell growth and survival in either cell line.

Furthermore, IPA revealed the top 10 dysregulated pathways in HCT 116 cells, of which only the EIF2AK1 (HRI) pathway was activated and the Xenobiotic Metabolism AHR Signaling Pathway was deactivated, with no other important pathways affected. Gene ontology study of enriched DEGs revealed potential roles in transcription regulation, kinase activity, transport, and enzymatic activities. However, none of enriched DEGs were directly engaged in the regulation of cell growth or viability.

Overall, the new medium formulation did not significantly affect the signaling pathways and/or genes that regulate cell growth and survival in the HCT 116 and U-2 OS cell lines. However, a small subset of genes in HCT 116 cells exhibited aberrant regulation in the new peptone-free medium, warranting further investigation—particularly in the context of cell culture for fundamental research applications and drug development.

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