

**GENE EXPRESSION PROFILES IN VIRUS PRODUCING AND LOW/NON VIRUS
PRODUCING EBV-BAC CONTAINING CELL LINES**

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Anna Lucas

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This thesis was presented

by

Anna Lucas

It was defended on

December 14, 2009

and approved by

Jeremy Martinson, DPhil, Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Frank Jenkins, PhD, Associate Professor
Department of Pathology
School of Medicine
University of Pittsburgh Cancer Institute

Thesis Advisor:

David T. Rowe, PhD, Associate Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

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Anna Lucas, M.S.

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Our lab studies Epstein-Barr virus (EBV); therefore, we need a supply of cells that steadily produce virus for use in our experiments. Currently virus harvesting is unpredictable, as transfection of 293SL cells with a given Bacterial Artificial Chromosome (BAC) may produce cell lines that vary widely in the amount of infectious virus produced, with most lines producing no virus at all. Using quantitative real time PCR we quantified EBV mRNA expression pertaining to 16 specific targets including latent, lytic, and promoter transcripts. This was to determine if there was a correlation between the pattern of virus gene expression and the capacity of a cell line to produce virus. Such a correlation could be used to develop a screening assay predictive of a cell line's potential for virus production. We found that the genes most useful for creating a PCR-based screening assay were the genes belonging to the EBNA3 family. The public health significance of the steady production of Epstein-Barr virus would be that experiments could be conducted on quicker time tables, which in turn may increase the rate of knowledge obtained about EBV.

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PREFACE

I would like to thank everyone in Dr. Rowe's lab for all of their help and support. Dr. Monica Jo Tomaszewski-Flick, Laura R. Wasil, Michael L. Davies, and Shushen Xu have been invaluable with their knowledge. Thanks to Holly Bilben and Lisa Mathews for their continued support and laughter. Thank you to my committee, Dr. Jeremy Martinson, Dr. Frank Jenkins, and Dr. Rowe for all of your help. I also give a huge thank you to Dr. Wilson for taking time out of his busy schedule to help me with my statistics.

1.0 INTRODUCTION

1.1 DESCRIPTION OF THE PROBLEM

Our lab studies Epstein-Barr virus (EBV); therefore, we need a supply of cells that steadily produce recombinant virus for use in our experiments. We use 293SL cells transfected with bacterial artificial chromosome (BAC) constructs containing wild type (B95.8) and mutant EBV for virus production. Currently virus harvesting is unpredictable, as transfection of 293SL cells with a given BAC produces cell lines that vary widely in the amount of infectious virus produced, sometimes even producing no virus at all. Our virus production is also inefficient in that it can take 1 to 2 months to generate enough cells to determine if the cell lines produce virus. This inefficiency wastes time and resources and significantly slows down the genetic analysis of EBV gene functions.

In this study we analyzed differences in viral gene expression between virus producing cells and low and non-virus producing cells. Our goal was to determine whether there was a predictive difference between producing and low/nonproducing cell lines that could allow us in the future to rapidly screen and identify which 293SL-based cell lines are likely to be good virus producers.

In order to characterize Epstein-Barr viral mRNA for 16 targets in 293SL EBV BAC containing cell lines, we quantified mRNA expression pertaining to 16 specific targets including latent, lytic, and promoter transcripts. The latent targets included the nuclear genes EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C and the three latent membrane protein genes

LMP1, LMP2A and LMP2B (1). The lytic targets included BZLF1, a lytic switch gene (2); the Bam HI A fragment of genome that is expressed during the lytic phase of EBV infection but is deleted in the B95.8 EBV virus (3), and BMLF1, a lytic early protein (4). The promoter sequences included the promoter sequences for Cp (C2:W1 nonproductive, a splice variant that produces EBNA2 transcripts and C2:W1 productive, a splice variant that produces EBNALP transcripts) and Wp (W0:W1 nonproductive, a splice variant that produces EBNA2 transcripts and W0:W1 productive, a splice variant that produces EBNALP transcripts), active during latency III, and the promoter sequence of FQ which becomes active during the switch from latency III to the lytic cycle (5). We determined how much of each transcript was being produced in both producer and low/nonproducer 293SL EBV cell lines and analyzed the results for trends that may be occurring in expression.

1.2 EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) was discovered by Denis Burkitt and Anthony Epstein. Burkitt noticed children in Kampala presenting with odd jaw tumors that he had not previously encountered. He surveyed sub-Saharan Africa and found jaw tumors in children living in areas endemic to malaria. Epstein biopsied the jaw lymphomas and discovered a new herpesvirus, Epstein-Barr virus (6).

Epstein - Barr virus is a gamma herpesvirus that replicates and causes latency in lymphoblastoid cells. Another gamma herpesvirus, Kaposi Sarcoma-associated Herpesvirus, causes lytic infections in epithelial or fibroblast cells as EBV does (6). The EBV genome is 184

kbp linear double-stranded DNA that immediately circularizes upon entry into the host nucleus (6).

Approximately 90% of the world's population is infected by Epstein–Barr virus (7). In developing countries primary EBV infection usually occurs at an early age, typically before age 10 and tends to be asymptomatic (7, 8). In developed countries EBV infection more often occurs during adolescence or young adulthood where the severity of the symptoms is increased, possibly resulting in tonsillitis, fever, malaise, swollen lymph nodes and infectious mononucleosis (7, 8). The most severe possible complications include a swollen/ruptured spleen, hepatitis, and chronic active EBV infection. EBV is also associated with the origins of Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinomas in immunocompetent carriers, as well as B cell lymphomas in immunosuppressed people (8).

EBV is spread orally. The initial EBV infection might occur either in squamous epithelial cells, like the tonsils, or in resting B lymphocytes near the tonsils (8). This is currently debatable. From infectious mononucleosis patients' tonsillar tissue, researchers have not found any evidence for epithelial replication. Small numbers of plasmacytoid B cells that express early lytic antigens are detected near crypt epithelium (6). However, there is evidence of epithelial lytic replication in AIDS patients with oral hairy leukoplakia and in healthy carriers' throat washings (6, 9). There is also evidence that EBV infection of epithelial cells requires initial infection of B cells. This may be resolved by the fact that epithelial cell infection is 1000 times more efficient if the virus first binds a resting B cell next to a permissive epithelial cell, implying that the presence of both B cells and epithelial cells are required for infection (6, 10).

Once in B lymphocytes EBV establishes a latent infection (8, 11). There are currently two models for primary B cell infection and latency that have been proposed from the

examination of tonsillar B cells taken from infectious mononucleosis patients. The first model is that EBV infects naïve B cells and causes them to express the latency III program (7). Latency III includes the viral expression of EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C, LMP1, LMP2 and EBV small encoded RNAs (EBERs) (12). The B cells then continue to express the latency I program (viral expression of EBNA1 and EBERs) while they cross through the germinal center. The infected B cells are protected in the germinal center by viral expression of the latency II program, which is expression of EBNA1, LMP1, LMP2 and EBERs (7, 12). When the B cells mature into resting memory B cells they switch to latency 0 (no EBNA expression at all), only expressing EBNA1 at the rare times when these resting B cells are dividing (7, 12). The other model proposed is that memory B cells can be directly infected by EBV in the germinal center during infectious mononucleosis (7).

1.3 THE ROLES OF EPSTEIN-BARR VIRUS GENES

1.3.1 Wp and Cp promoter Splice Regions

The W region of the EBV genome is composed of 6-12 repeats of a 3072bp sequence that encodes Wp, the initial promoter for EBV mRNA transcription in latently infected lymphocytes (LCLs). When Wp is activated transcripts encoding EBNA1P and EBNA2 are the first made. EBNA1P and EBNA2 are spliced from the same primary transcript and alternative splice site selection determines if the EBNA1P open reading frame can be translated (6). The W0:W1 splice produces EBNA1P, while the W0:W1 splice is nonproductive for EBNA1P but still encodes EBNA2 (6). Over time EBNA2 and EBNA1P cooperate to activate the upstream viral

Cp promoter, which causes the gradual change from the Wp promoter usage to the Cp promoter usage as Cp transcription represses Wp activity (1, 6, 9).

When Cp replaces Wp function the C2:W1 nonproductive splice produces the EBNA2 transcript, while the C2:W1 productive splice creates the EBNA1P transcript. Preceding the EBNA2 start codon is an alternative splice site that can lead to expression of EBNA3A, EBNA3B, EBNA3C, and EBNA1 instead of the EBNA2 transcript (6). When the Cp and/or Wp promoters are switched on and the Qp promoter is switched off during latency type III, all of the EBNA2s are translated from large transcripts that comprise 60% of the EBV genome (7).

After EBV infection of 293 epithelial cells both Wp and Cp are active but become methylated while the Qp promoter region stays unmethylated, making the epithelial cells exhibit the restricted latency patterns of type I and type II classically observed in nasopharyngeal carcinoma (13).

1.3.2 EBNA2 and EBNA1P

By 32 hours post infection EBNA2 and EBNA1P are at their maintenance levels in LCLs (6).

EBNA2 is critical for B cell transformation and is responsible for making the resting B cell go from cell cycle G0 to G1 (1,9). EBNA2 and EBNA1P coactivate the LMP1, LMP2A, and LMP2B promoters (6).

EBNA2 has been considered functionally similar to Notch because of their mutual interaction with RBP-Jκ and though they do function similarly, Notch tends to induce more developmental and differentiation signal upregulation while EBNA2 functions more in cell cycling, proliferation, and upregulation of apoptosis when antiapoptotic elements like LMP1 are missing (14). EBNA2 has also been shown to delay growth in transfected 293 cells by activating

expression of p53-regulated genes (15). While both EBNA2 and EBNA1 are associated with the nuclear matrix they differ in that EBNA1 is partially located to the cytoplasm but EBNA2 is associated with the nucleoplasm and chromatin of cells(6).

1.3.3 EBNA1

EBNA1 is responsible for tethering the EBV episome to cellular DNA to ensure that viral DNA replication occurs along with cellular DNA replication, and is also the only EBNA that continues to be transcribed when cells enter lytic infection (1, 6). Its distribution is widespread among mitotic chromosomes (6). In addition EBNA1 is a transcription factor that can bind downstream of one of its own viral promoters, Qp, to regulate its own expression (1, 16). EBNA1 can also upregulate the viral Cp promoter and the viral LMP1 promoter (1). When the Cp and Wp promoters are switched off during latency types I or II, EBNA1 is expressed by the Qp promoter (7).

1.3.4 The EBNA3 Family

The EBNA3 family of three related proteins, like EBNA2, is also involved in the process of B cell immortalization but only EBNA3A and EBNA3C are crucial for the process. The EBNA3s are transcriptional regulators that can upregulate cellular and viral expression. Overexpression of EBNA3A can mitigate strong upregulatory signals from EBNA2, and along with EBNA3C, can repress the Cp promoter and stop LCL growth (1, 9, 13, 15). LCLs have very few copies of EBNA3 mRNA but their proteins are very stable at high levels (6). Similar to EBNA2 the EBNA3 family is also associated with the nuclear matrix, chromatin, and nucleoplasm (6).

1.3.5 LMP1

LMP1 is the main transforming protein of EBV and has clear oncogenic properties (17, 18). It is fundamentally an active tumor necrosis factor receptor and functions as a mimic to cellular CD40 to provide growth, differentiation, and survival signals to B cells infected with EBV (6, 17). Expression of LMP1 prevents apoptosis by downregulating bcl2 expression and being the principal activator of NF- κ B in LCLs, as well as modulating the immune system (6, 18, 19). LMP1 shares a bidirectional promoter with LMP2B.

LMP1 localizes to the plasma membrane in LCLs and forms large aggregations. In epithelial cells it is more sporadically distributed throughout the surface and internal cytoplasmic membranes (6). LMP1 alters the growth patterns of both primary B lymphocytes and epithelial cells in both infected and transfected cells (6). It also blocks the normal process of differentiation in epithelial cells (19).

1.3.6 LMP2A and LMP2B

LMP2A and LMP2B are two isoforms of the LMP2 gene but they have unique 5' first exons and share the remaining eight 3' exons (20, 21). LMP2A contains an N-terminal, signaling domain which acts as a B cell receptor mimic involved in transformation of B cells, inhibition of apoptosis, and B cell survival and proliferation in the absence of B Cell Receptor (BCR) signaling. LMP2A also blocks some aspects of cellular BCR signaling, including the proapoptotic pathway that is activated by BCR crosslinking in the absence of co-stimulatory signals (6, 17, 21). LMP2A in LCLs colocalizes with LMP1 and interacts with lipid rafts where LMP2A can increase LMP1 signaling (6, 22, 23). Since LMP2B does not contain a signaling

domain but retains sequence homology to LMP2A, it has been implicated as a negative regulator for LMP2A (20).

1.3.7 FQ

The FQ transcript contains both the Fp and the Qp promoters. During both latency I and II Cp and Wp are suppressed while Qp initiates EBNA1 transcription (9). Fp is upstream of Qp and is not active during latency but becomes active early in the switch from latency III to the lytic cycle (5).

1.3.8 BMLF1 and BZLF1

BMLF1 is expressed as an early protein during the process of lytic reactivation. A large proportion of cytotoxic T cell activity is directed against the BMLF1 protein during primary EBV infection (4). BMLF1 functions in exporting specific viral mRNA from the nucleus to the cytoplasm (24). It is a gene that is essential for EBV replication and RNA stabilization (6).

BZLF1 is an immediate early gene that is often used to deliberately induce lytic replication in epithelial cells (6). BZLF1 is able to initiate the lytic cycle as well as function in replication and can also arrest the cell cycle (2). BZLF1 can induce expression of Rta, another viral lytic activator for late lytic genes (2). EBV DNA replication is dependent on both BMLF1 and BZLF1 (6).

1.3.9 Bam HI A Region

The BamHI A region transcripts possibly play a role in tumor formation because they have been found to be expressed at high levels in nasopharyngeal carcinoma and other EBV associated malignancies. Their function is not fully understood but may be important for virus persistence and involve modifying signal transduction pathways (1, 7). There is a deletion in this region in the B95.8 virus that we used for our experiments but the deletion has no noticeable effects on in vitro EBV mediated B cell immortalization (3, 25).

1.4 PREVIOUS STUDIES

While there are previous studies mentioning virus production in transfected epithelial cells, there are no studies that are trying to determine if the latent EBV gene transcription in the transfected 293 cells is having an effect on the amount of virus being produced (26, 27, 28).

2.0 MATERIALS AND METHODS

2.1 STUDY OVERVIEW

Wild types and mutant constructs containing FLAG tags and a hygromycin resistance marker were previously made and inserted into bacterial artificial chromosomes (BACs). The BACs were then transfected into 293SL cells which are an adherent epithelial cell line.

Transfected cells expressing EGFP have been selected with hygromycin. Only the cells containing virus BACs survived the selection. The selected cells were cultured and green colonies were grown into cell lines. Cells lines are induced to produce EBV by transfection with BZLF1 and gB-expressing plasmids.

Following incubation of transfected cells for 5 days at 37°C, supernatants were harvested and used for titration. The supernatant was layered over Raji B cells to determine infectivity. If infectious virus was detected via the expression of EGFP in the Raji cells, the cell line was designated as a virus producer (sometimes a low virus producer, depending on the viral titer). If virus was not detected in the cellular supernatant the cell line was designated as a nonproducer. Our lab prefers to use GIU/mL. Any cell line making less than 10⁵ GIU/mL would be considered a low producer and there were some cell lines that did not produce virus at all.

Table 1: Producer status of Cell Lines.

Producers		
EBV	Virus Phenotype	Green Inducing Units (GIU)/ml
E2089.0/I4	LMP2A+/LMP2B+	1.9×10^6
E2089.1/D2	LMP2A+/LMP2B+	1.8×10^5
E2089.3/B2	LMP2A+/ Δ LMP2B	3.1×10^6
E2089.4/#1	LMP2A+/LMP2B ^{NEG}	4.1×10^6
E2525.1/D1	Δ LMP2A/LMP2B+	5×10^5
E2525.2/C3	Δ LMP2A/LMP2B+	8.6×10^5
Low Producers		
EBV	Virus Phenotype	Green Inducing Units (GIU)/ml
E2089.0/E2	LMP2A+/LMP2B+	10^2 /ml
E2089.4/O4	LMP2A+/LMP2B ^{NEG}	10^3 /ml
Non Producers		
EBV	Virus Phenotype	
*E2089.1/C4	LMP2A+/LMP2B+	
*E2089.3/C1	LMP2A+/ Δ LMP2B	
*E2525.1/J1	Δ LMP2A/LMP2B+	
*E2525.2/B5	Δ LMP2A/LMP2B+	
*E2525.3/C3	Δ LMP2A/ Δ LMP2B	
*E2525.3/D6	Δ LMP2A/ Δ LMP2B	

Once producer, low producer, or nonproducer status was determined and the cells were grown out into separate clonal lines, cells from each line were counted with a hemocytometer. The appropriate amount of cell suspension was removed to make aliquots containing 2 million cells each and then centrifuged. At least 3 cell pellets from each cell line were collected at the same time. Each pellet was put in RNAlater and temporarily stored in the -20°C freezer until extraction. These pellets were phenol/chloroform extracted within a week of being put into the -20°C freezer.

After all of the pellets were processed the entire RNA product was reverse transcribed using random hexamers and reagents according to the protocol supplied with the Applied Biosystems GeneAMP RNA PCR kit. The cDNA product was used in a series of real time PCR experiments for β 2m (a cellular housekeeping gene), LMP1, LMP2A, LMP2B, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, FQ, BZLF1, BMLF1, and the Bam HI region. The real time quantitative PCR results were normalized to the β 2m expression. The normalized data was plotted on a log scale and analyzed within each cell line using an unpaired, two-tailed, Student's t test to look for variation within each individual cell line. In order to be able to compare data both between pooled cell lines and also between pooled producers and pooled low/nonproducers the data was converted to log base 10 data and analyzed with two way unpaired ANOVA with equal sample sizes.

The major limitations of this study were mostly confined to cell line production. It took five months to procure all of the cell lines necessary for the study. Samples from the 2525.4 cell line were not analyzed as cells with these BACs had not sufficiently grown into stable cell lines. There were also instances, such as the 2089.0 and 2089.4 cell lines, where the isolated lines were consistently producers and true nonproducers were not isolated. Low producers were used for comparison instead. The reverse was also true, as in the 2525.3 cell line, where the cell lines were non or poor producers and examples of producer cell lines were not available.

This study was the first to examine the effect of latent EBV gene transcription on virus production in transfected 293 cells. There was no previous work with which to directly compare our results. This transfected 293 system is quite different from EBV-immortalized B cells or EBV-infected primary epithelial cells.

2.2 CELL LINES AND VIRUS

293SL cells were transfected with mutant and wild type EBV constructs in bacterial artificial chromosomes. The 293SL cells were cultured in DMEM, 60 µg/mL penicillin, 200 µg/mL streptomycin, 2mM L-glutamine, 10% heat inactivated FBS, and 75 µg/mL hygromycin. The Epstein-Barr viruses used for all experiments and constructs were based on the B95.8 virus strain.

Figure 1 depicts all of the constructs used during this project. Exon 1 starts the message that encodes LMP2A; Exon 1' starts the message that encodes LMP2B. 2089.0 is wild type B95.8 EBV. 2089.1 is a wild type virus with a flag tag in loop 11 of the LMP2 ORF, represented by the green diamonds in the remaining exons of LMP2A/LMP2B. We did not have any 2089.2 cells as we do not have this virus BAC in production. 2089.3 is a virus with a LoxP deletion in the LMP2B promoter region. The LoxP sites are represented by the blue lollipop structures. The M to C point mutations in lines 2089.4 and 2525.4 indicate mutations of initiator methionine to cysteine in order to eliminate the production of LMP2B protein without deleting Exon 1'. These BACs were made because the deletion in the 2089.3 BACs might interfere with the expression of LMP1 from the LMP1/LMP2B bidirectional promoter shown by the opposing black arrows connected by a line in the constructs.

The 2525 cell lines all have LoxP deletions in the LMP2A region (done by the Hammerschmidt group). 2525.1 is wild type in the LMP2B region. 2525.2 is also wild type but has LoxP sites around the LMP2B promoter region. 2525.3 is LoxP deleted and lacks the first exons of both the LMP2A and LMP2B messages. As mentioned previously we did not have any of the 2525.4 BAC cell lines available for this study.

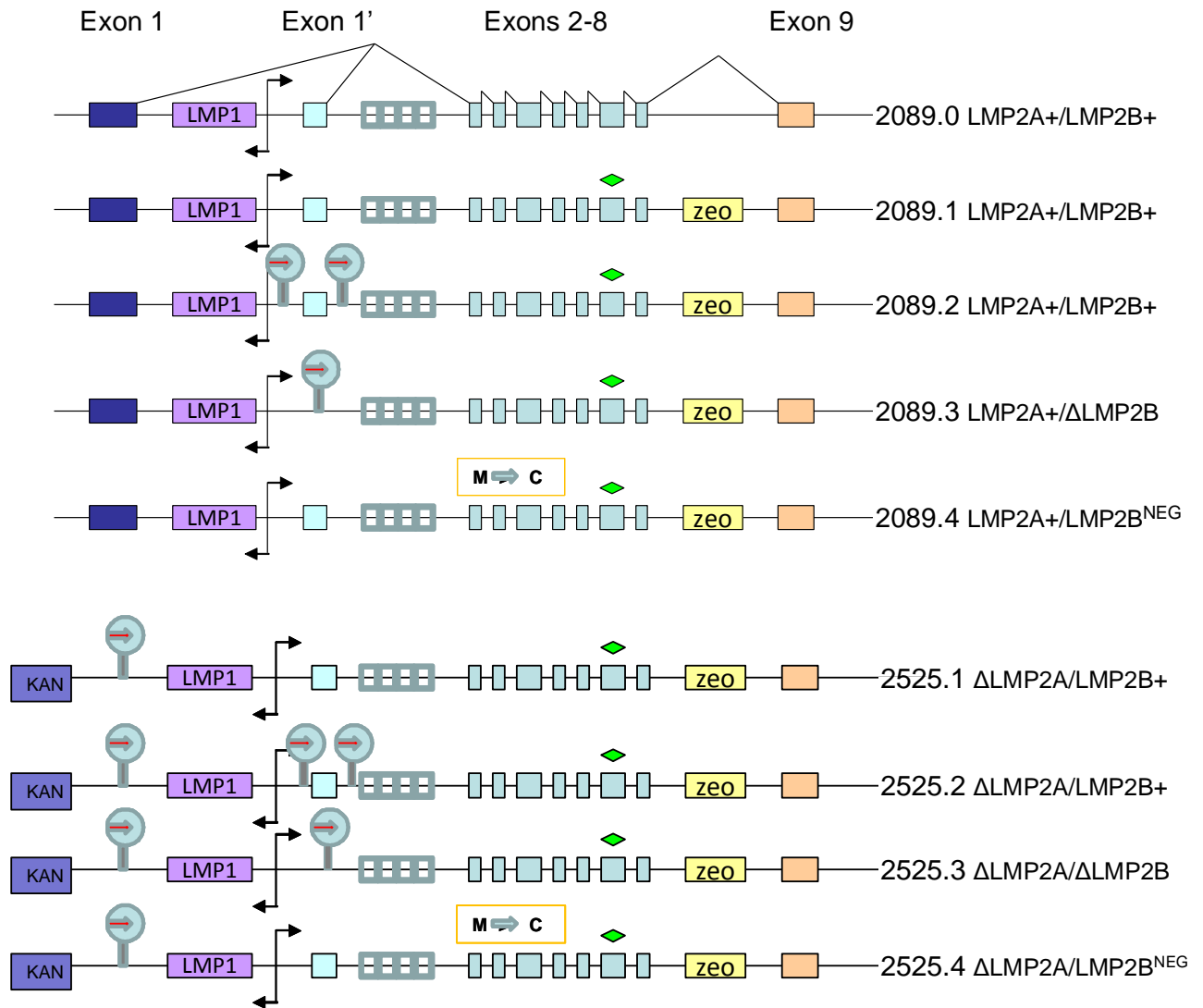


Figure 1. Constructs used during this project.

2.3 RNA EXTRACTIONS

Two million cells of each sample being analyzed were pelleted and at least 3 separate pellets of each sample were analyzed. Five hundred μL of RNAlater ammonium sulfate based RNA

stabilization buffer (QIAGEN) was added to each pellet and the pellets were stored at -20°C. All RNA from cells was extracted within a week of being pelleted.

In order to extract the RNA from the cell pellet the RNAlater was removed and one mL of TRIZOL reagent was added and repetitively pipetted to lyse the cells. The homogenized samples were incubated for 5 minutes at room temperature. 0.2 mL of chloroform was added to each sample and shaken vigorously by hand for 15 seconds. The samples were incubated at room temperature for 3 minutes then centrifuged at 12000rpm at 5°C for 15 minutes. The upper colorless aqueous phase where the RNA remained was drawn off and put into a new tube. 0.5mL of isopropanol was added to each aqueous sample and repetitively pipetted to precipitate the RNA. The samples were then incubated at room temperature for 10 minutes and subsequently centrifuged at 12000rpm at 5°C for 10 minutes. The supernatant was removed leaving behind an RNA pellet.

The pellet was washed once with 1 mL of a 75% solution of ethanol and RNase free water. The tubes were gently agitated then centrifuged at 7500rpm at 5°C for 5 minutes. The supernatant was again removed and each pellet was air dried for 5 minutes. 50 µL of DEPC-treated RNase free water was added to each sample with repetitive pipetting to help the RNA dissolve and incubated at 55°C for 10 minutes. After the protocol was finished all extracted RNA was stored in a -80°C freezer until needed. All RNA samples were measured for quantity and quality of RNA using a spectrophotometer.

2.4 REVERSE TRANSCRIPTION

All of the reagents used except for the RNA template and the DEPC-treated RNase free water came from the Applied Biosystems GeneAMP RNA PCR kit. For a single reverse transcription reaction a master mix of 5 μL RT Buffer, 11 μL MgCl_2 , 10 μL of 2.5mM dNTPs, 2.5 μL random hexamers, 1 μL Multiscribe Reverse Transcriptase, 1.25 μL RNase inhibitor, and 9.25 μL DEPC-treated RNase-free water were mixed. Multiple reactions were done at the same time and aliquotted into mini Eppendorf tubes. 10 μL of RNA template was added (for a total volume of 50 μL) with pipetting to mix the solution, then briefly centrifuged before being put into the ABI 9700 thermal cycler. A no-template no-reverse template control was also included. The temperatures programmed into the cycler were 50°C for 30 minutes, 90°C for 10 minutes and 4°C for the duration of the run until the product was put into the -80°C freezer.

2.5 REAL TIME ABSOLUTE QUANTIFICATION PCR FOR MRNA

All of the primers used for the real time PCR reactions are shown in Table 2, created and generously permitted to display by Michael L. Davies, PhD student in Dr. Rowe's lab. For a single real-time PCR reaction a master mix of 2.5 μL PCR Buffer II, 2.5 μL MgCl_2 , 2 μL of 2.5mM dNTPs, 1 μL 20 μM 5' primer, 1 μL 20 μM 3' primer, 0.5 μL 10 μM probe, 0.5 μL ROX, 0.125 AmpliTaq gold, and 12.875 μL MilliQ water were mixed in a tube. Multiple reactions were done at the same time and aliquotted into 96 well PCR plates. 2 μL of cDNA template was added for a total volume of 25 μL in each well and then the plate was covered with optical film and centrifuged to remove air bubbles. The plate was placed in the ABI 7500

Taqman machine. The temperatures were 50°C for 2 minutes, 95°C for 2 minutes; 95°C for 15 seconds, 60°C for 60 seconds, repeated forty times; 60°C for 5 minutes; then 4°C for the duration of the run or until the plate was removed from the Taqman machine.

All real-time PCR experiments were carried out in triplicate for each mRNA target. Each mRNA target was quantified by comparison to pre-made standards containing known amounts of the target being analyzed. Negative, no template controls as well as a no-template, no-reverse transcription control were included for each target. RNA for the endogenous control gene β 2m (β 2- microglobulin) was also measured for each sample so that viral genes could be normalized to cell number.

Table 2. Primers used in the real time PCR experiments.

Transcript	Forward primer (5' to 3' sequence)	Reverse primer (5' to 3' sequence)	probe*
EBNA1	GAT TCT GCA GCC CAG AGA GTA GTC	TCG TCA GAC ATG ATT CAC ACT TAA AG	TCG TCG CAT CAT AGA CCG CCA GTA GAC
EBNA2	TAA CCA CCC AGC GCC AAT C	GTA GGC ATG ATG GCG GCA G	CAC CAC GTC ACA CGC CAG TGC TGG GT
EBNA3A	GAT TCT GCA GCC CAG AGA GTA GTC	CTT CTT CCA TGT TGT CAT CCA GG	CCC GGC CTG TCC TTG TCC ATT TTG
EBNA3B	GAT TCT GCA GCC CAG AGA GTA GTC	CCA CGC TTT CTT CAT TAT TCA GGT	TAG ACC GCC AGT AGA CCT GGG AGC AGA
EBNA3C	GAT TCT GCA GCC CAG AGA GTA GTC	CCA GGG TCC TGA TCA TGC TC	AAG ACC CAC CAT GGA ATC ATT TGA AGG A
LMP1	TCA TCG CTC TCT GGA ATT TG	TCC AGA TAC CTA AGA CAA GTA AGC AC	AGC ACA ATT CCA AGG AAC AAT GCC TGT C
LMP2A	CTA CTC TCC ACG GGA TGA CTC AT	GGC GGT CAC AAC GGT ACT AAC T	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
LMP2B	CGG GAG GCC GTG CTT TAG	GGC GGT CAC AAC GGT ACT AAC T	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
C2W1 non	TCC TGC ACG TGA GCA TCC T	TTC TAC GGA CTC GTC TGG GTT	TGA AGG CCC TGG ACC AAC CCG
C2W1 pro	TCC TGC ACG TGA GCA TGG G	TTC TAC GGA CTC GTC TGG GTT	TGA AGG CCC TGG ACC AAC CCG
WOW1 non	CCA GGA GTC CAC ACA AAT CCT A	TTC TAC GGA CTC GTC TGG GTT	TGA AGG CCC TGG ACC AAC CCG
WOW1 pro	CAG GAG TCC ACA CAA ATG GGA	TTC TAC GGA CTC GTC TGG GTT	TGA AGG CCC TGG ACC AAC CCG
BZLF1	TTC CAC AGC CTG CAC CAG T	AGC AGC CAC CTC ACG GTA GT	CAA CAG CCA GAA TCG CTG GAG GAA TGC G
BMLF1	CCT ACC TCG GCA TCG TTT GT	TCC GCG TCG CCT TTT GT	TGA CTG TCT TGT CCT GTA GGT CCC ACT TCT
Fp-Qp	CTT GAA AAG GCG CGG GAT A	GCG GTC TAT GAT GCG ACG AT	CCA AAC GCT CAT CCC AGG GAA GC

***All probes were conjugated with 5' FAM and a 3' TAMRA quencher**

2.6 REAL TIME ABSOLUTE QUANTIFICATION PCR FOR DETECTING B2M

For a single real-time PCR reaction a master mix containing 2.5 μL PCR Buffer II, 2.5 μL MgCl_2 , 2 μL of 2.5mM dNTP's, 1.25 μL $\beta 2\text{m}$ primer mix, 0.5 μL ROX, 0.125 AmpliTaq gold, and 14.125 μL MilliQ water was mixed in a tube. Multiple reactions were done at the same time and aliquotted into 96 well PCR plates. 2 μL of cDNA template was added for a total volume of 25 μL . The plates were covered with optical film then centrifuged to remove air bubbles. The plate was placed in the ABI 7500 Taqman machine. The temperatures were 50°C for 2 minutes, 95°C for 2 minutes; 95°C for 15 seconds, 60°C for 60 seconds, repeated forty times; 60°C for 5 minutes; then 4°C for the duration of the run or until the plate was removed from the Taqman machine. The median $\beta 2\text{m}$ value was 6.18.

2.7 TWO WAY UNPAIRED ANOVA WITH EQUAL SAMPLE SIZES

The 2 way ANOVA was carried out by Graph Pad Prism 5's InSite statistical package. This particular test performs 3 separate comparisons with the same input data sets. In one aspect it compares pooled producer data against pooled low and nonproducer data while disregarding which cell line the data came from. In another aspect it compares the pooled 2089 cell line groups against the pooled 2525 cell line groups while disregarding the producer versus low/nonproducer status. The final aspect of this statistic compares both producer vs.

low/nonproducer status against pooled 2089 cell lines and pooled 2525 cell lines to determine if there are differences occurring that are dependent on all four variables.

3.0 RESULTS

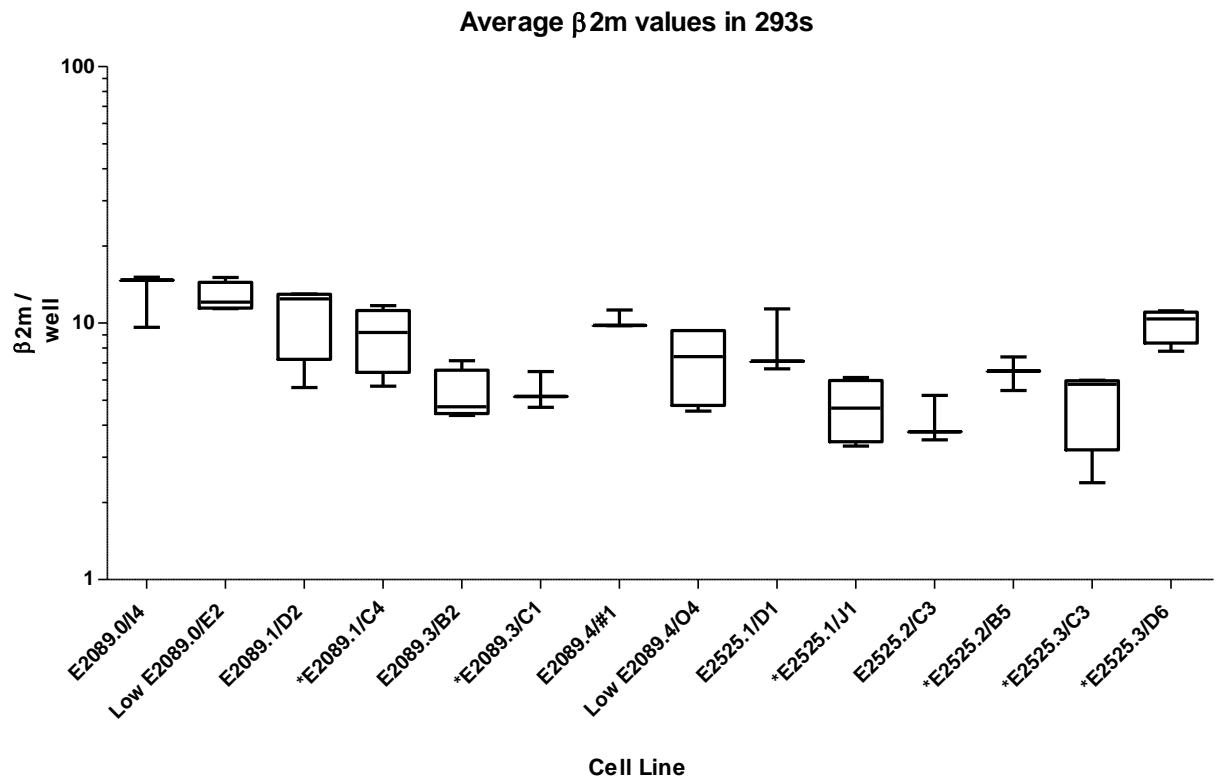


Figure 2. Average β 2m values measured by real time PCR (log scale).

Figure 2 shows the real time PCR measurement of the average β 2m values per cell line used in this study. The quantities range between 5 and 15 per well, showing little variability. The median β 2m was 6.18. In the Applied Biosystems Application Note it is mentioned that β 2m has been shown to have “relative stability across a number of tissues. (29)”

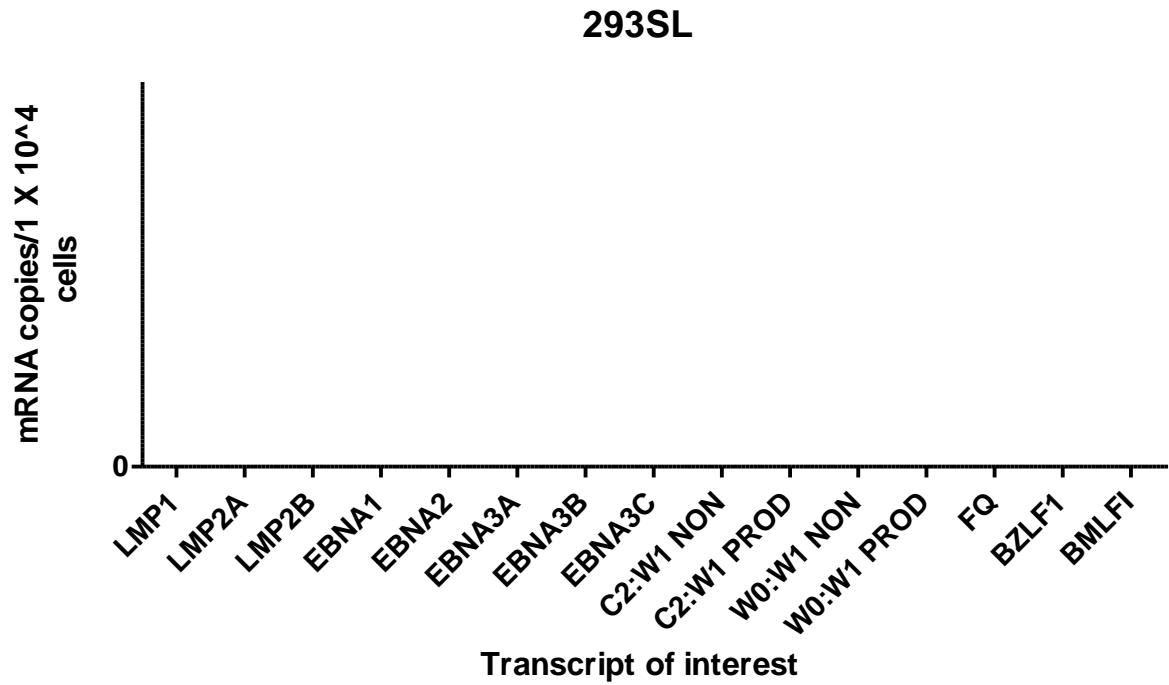


Figure 3. Untransfected 293SLs

Figure 3 shows the real time PCR results of 3 separate pellets, of 2 million cells each, of untransfected 293SLs. There was no transcription from any gene targets being measured.

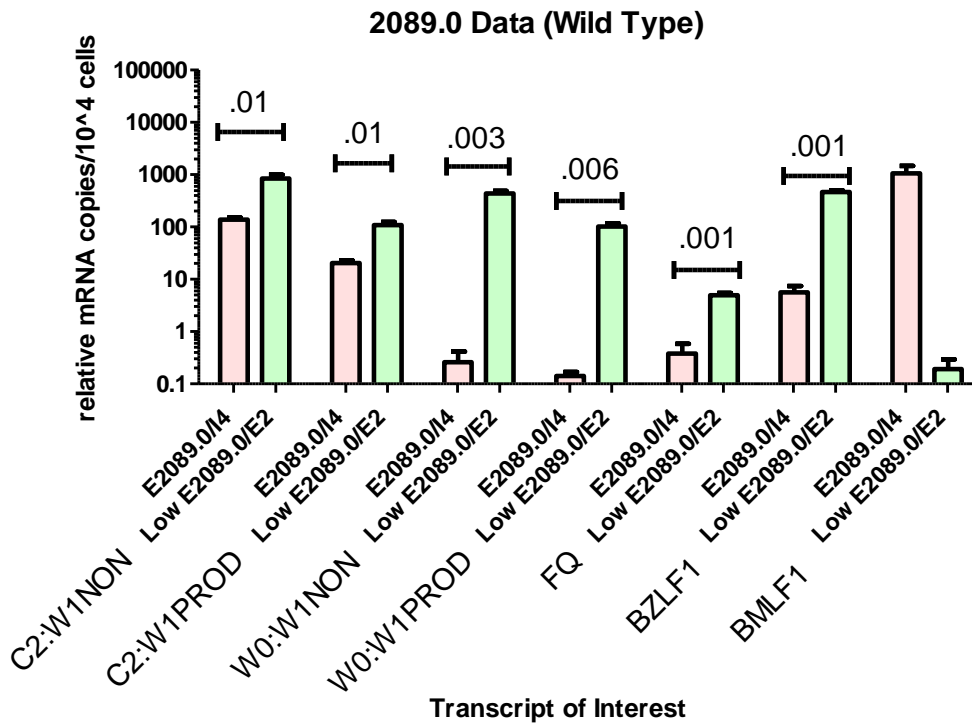
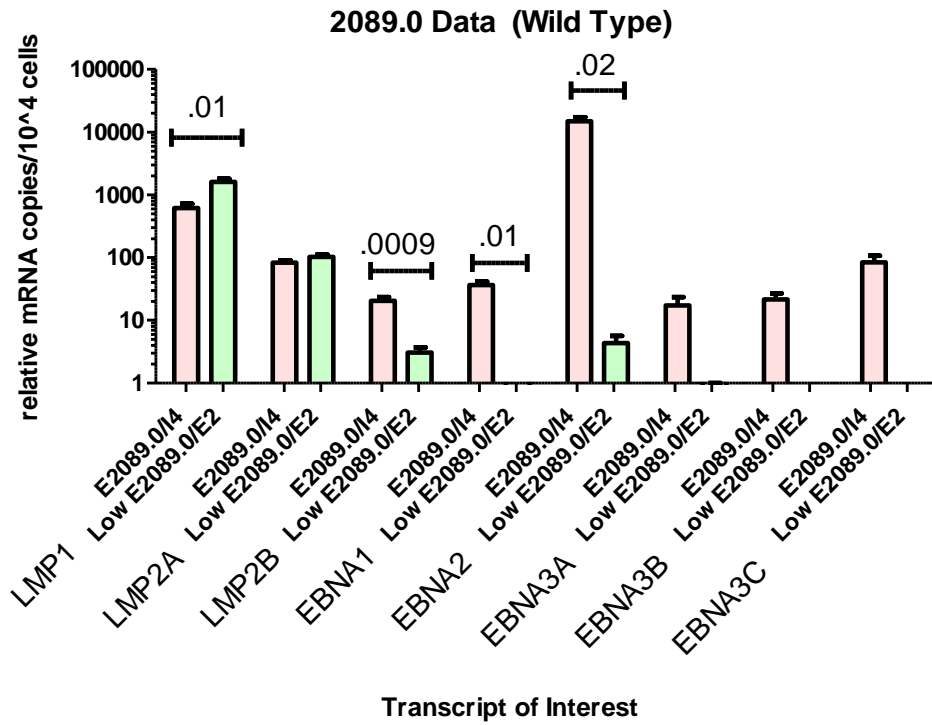


Figure 4. Gene transcription within the 2089.0 Cell Lines

Figure 4 depicts differences between the producer and low producer cell lines containing the 2089.0 BAC. The measurements of producers are shown in pink while the low producers are shown in green. Most of the differences were moderately statistically significant, such as within LMP1 ($p=0.01$) and EBNA2 ($p=0.02$). Highly significant statistical differences were detected between the W promoter regions, FQ ($p=0.001$), BZLF1 ($p=0.001$), and LMP2B ($p=0.0009$).

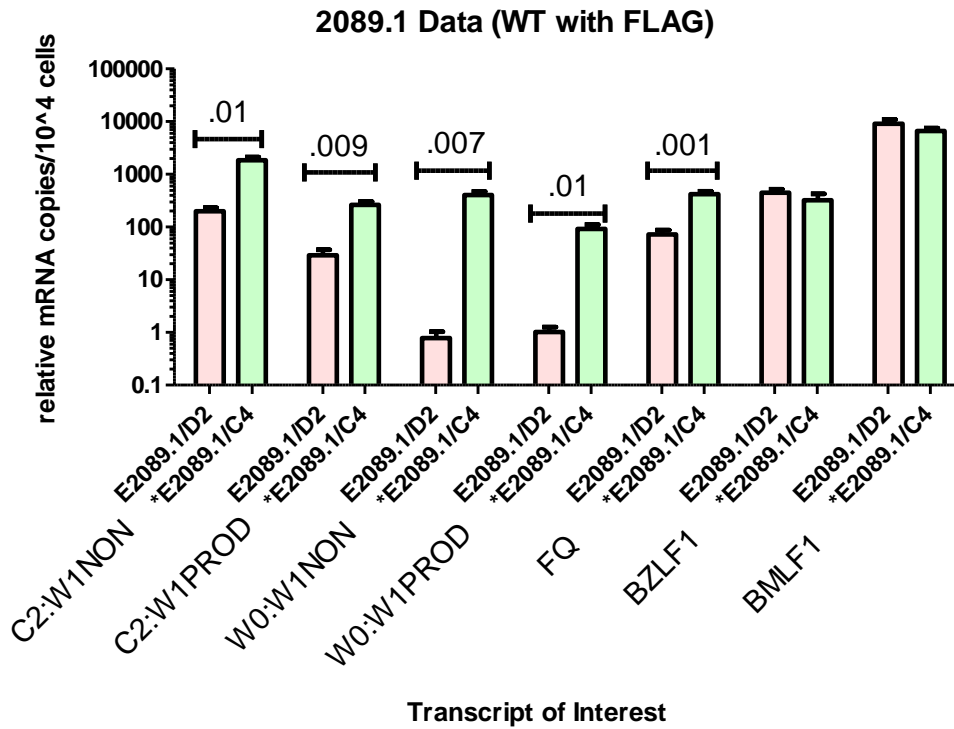
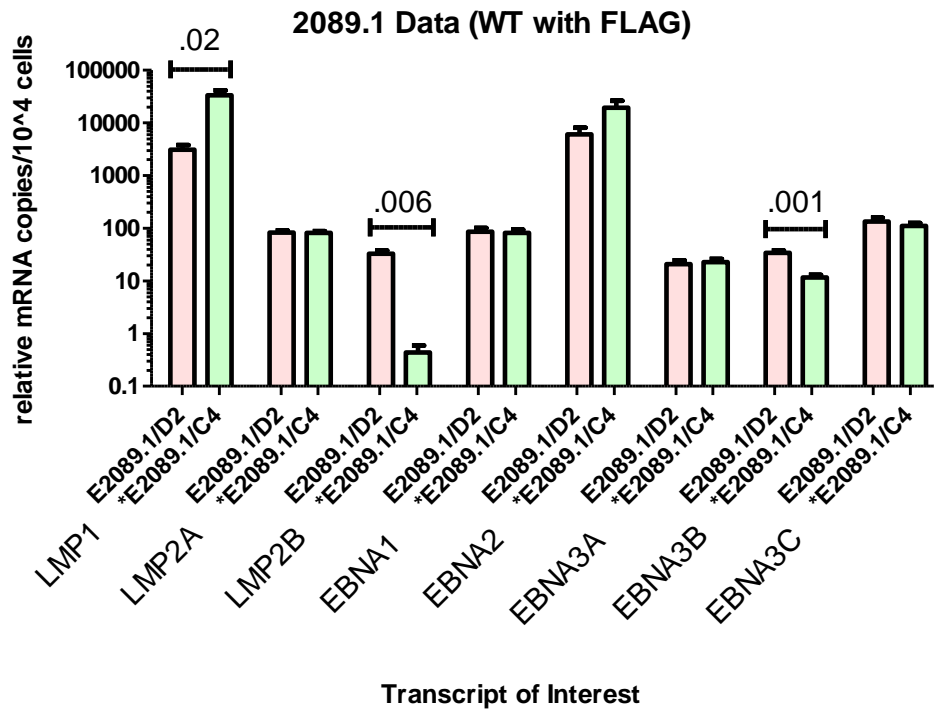


Figure 5. Gene transcription within the 2089.1 Cell Lines

Figure 5 depicts the differences between 2089.1 producers and nonproducers. Nonproducers are denoted with an asterisk preceding the name of the cell line in addition to the bar being colored green. Statistically significant differences were seen in LMP2B ($p=0.006$), EBNA3B ($p=0.001$), FQ ($p=0.001$), the productive splice of C2:W1 ($p=0.009$) and the nonproductive splice of W0:W1 ($p=0.007$).

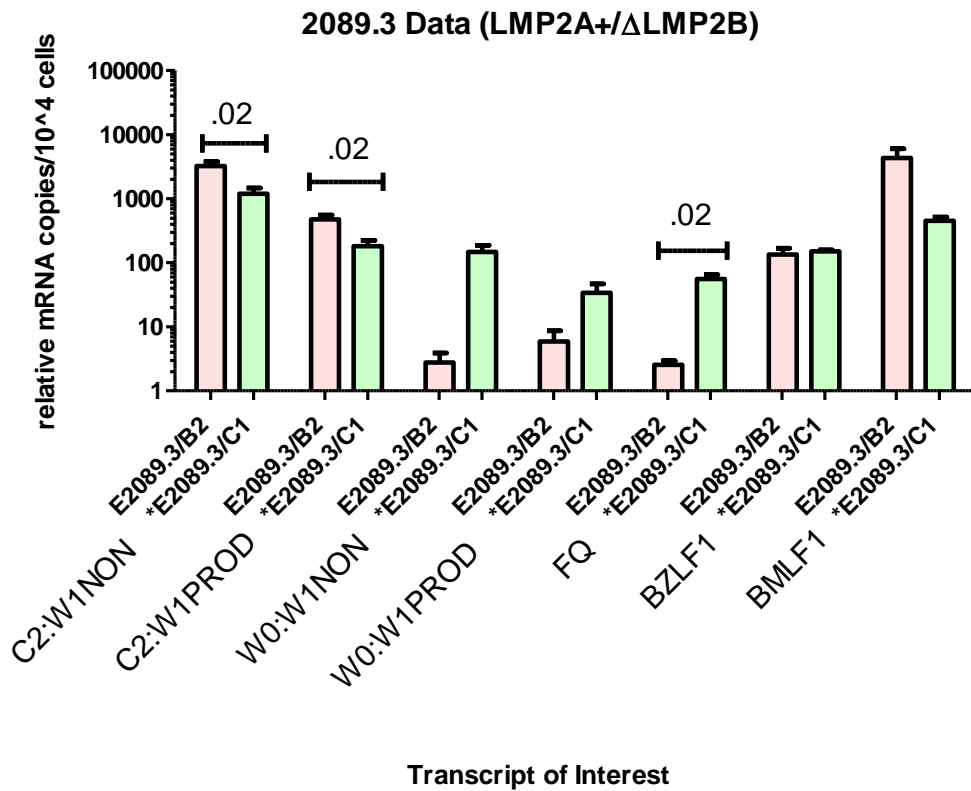
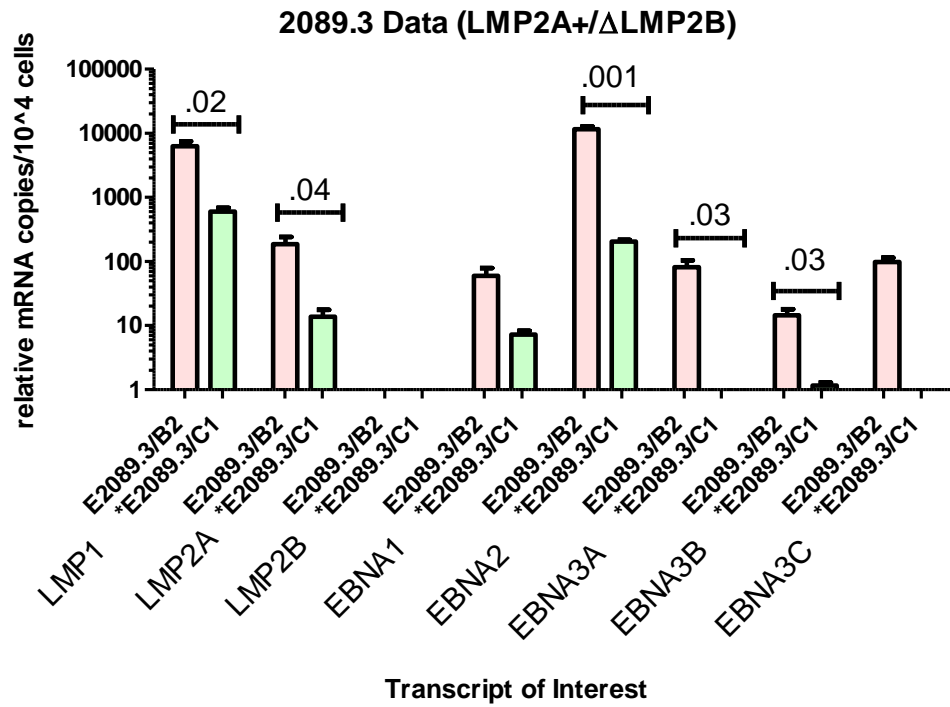
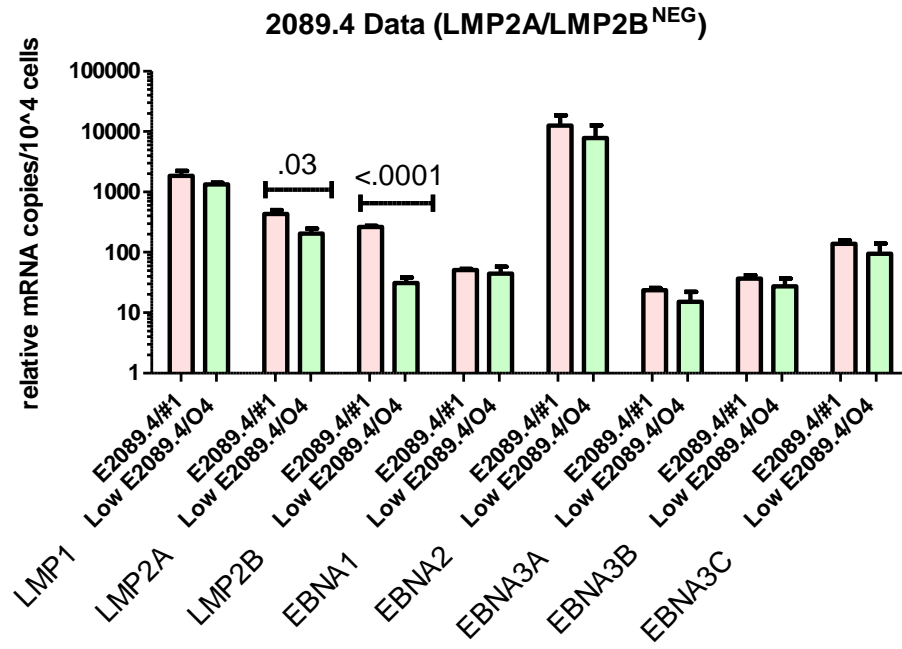
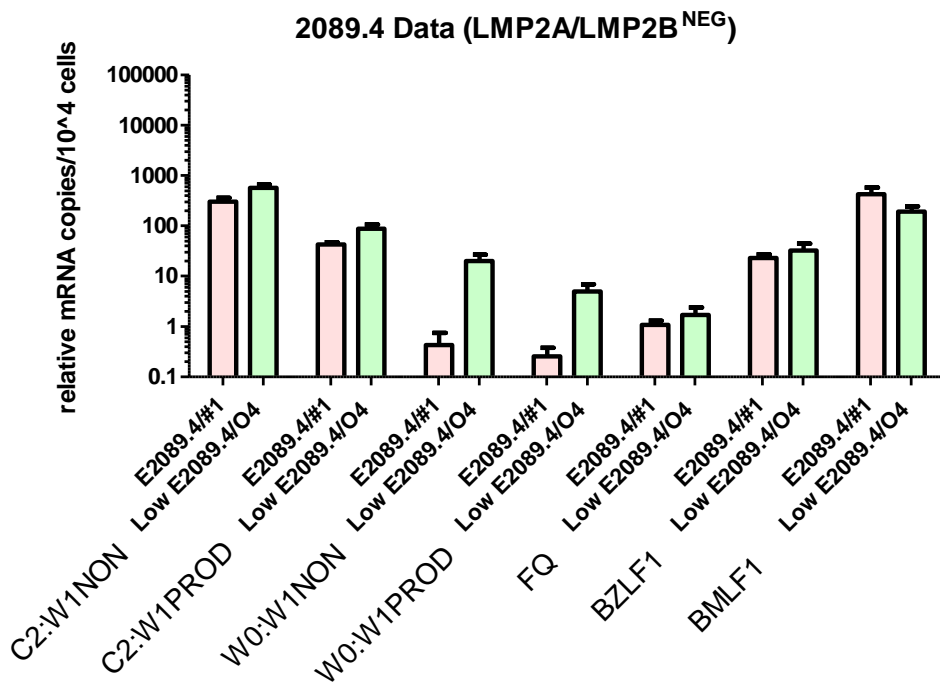


Figure 6. Gene transcription within the 2089.3 Cell Lines

Figure 6 depicts the differences between the 2089.3 producers and the 2089.3 nonproducers. Statistically significant transcript measurements were detected in EBNA2 ($p=0.001$). As expected the LMP2B gene transcript was not present because the LMP2B start for Exon 1' is LoxP deleted in this cell line.



Transcript of Interest



Transcript of Interest

Figure 7. Gene transcription within the 2089.4 Cell Lines

Figure 7 depicts the differences between the 2089.4 producer cell line and the 2089.4 low producer cell line. The 2089.4 cell lines express an LMP2B transcript but they fail to make an LMP2B protein because of the methionine to cysteine point mutation in Exon 2. A statistically significant difference was detected in the LMP2B gene ($p < 0.0001$).

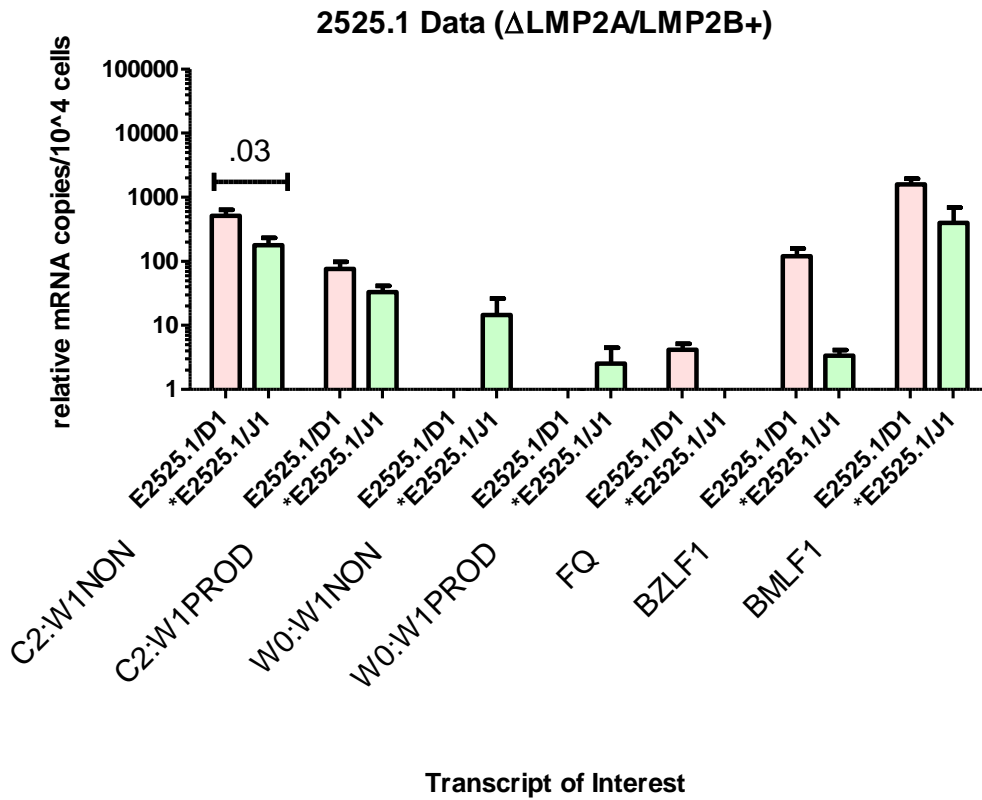
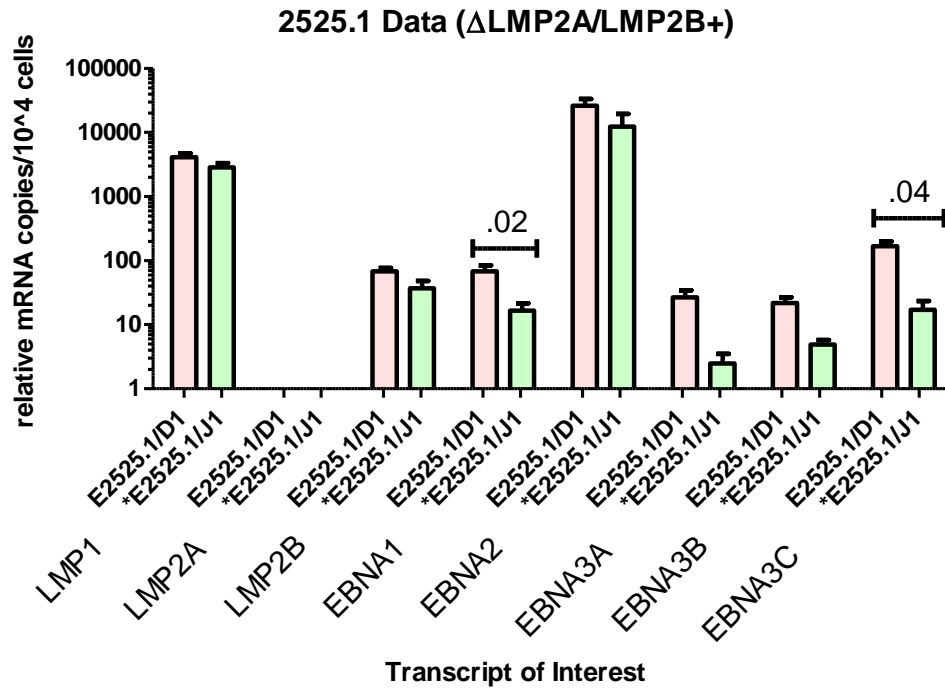


Figure 8. Gene transcription within the 2525.1 Cell Lines

Figure 8 depicts the difference between the 2525.1 producer cell line and the 2525.1 nonproducer cell line. None of the differences were highly significant. There was a lack of LMP2A transcripts in all of the 2525 cell lines as the LMP2A message start and Exon 1 is LoxP deleted in the 2525 cell lines.

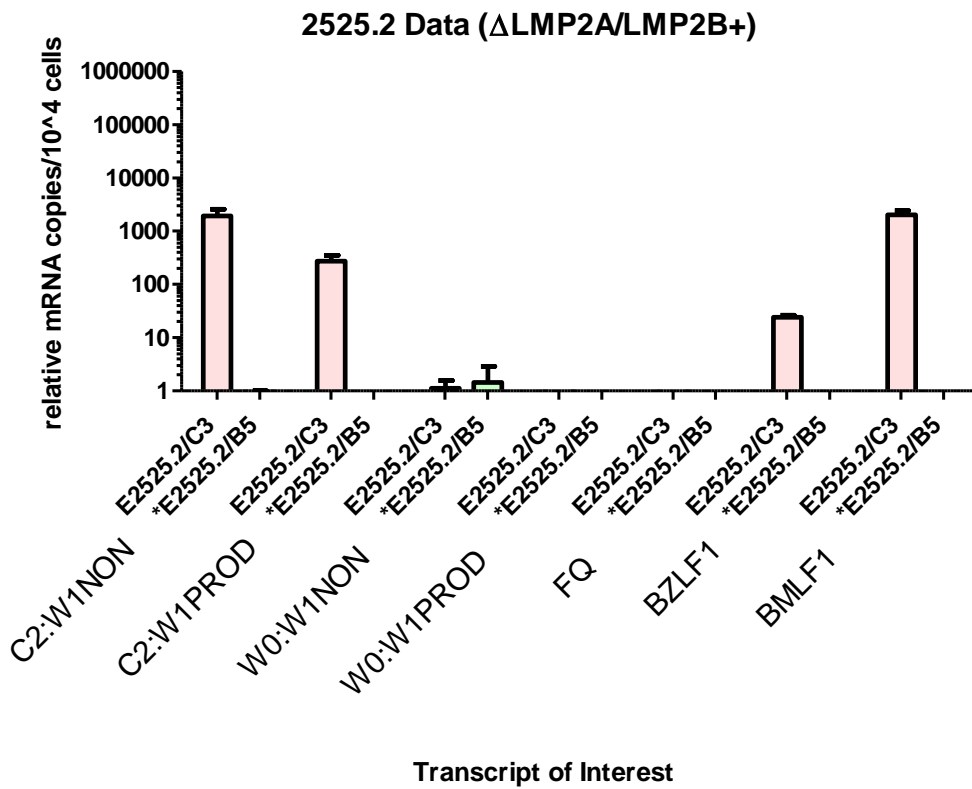
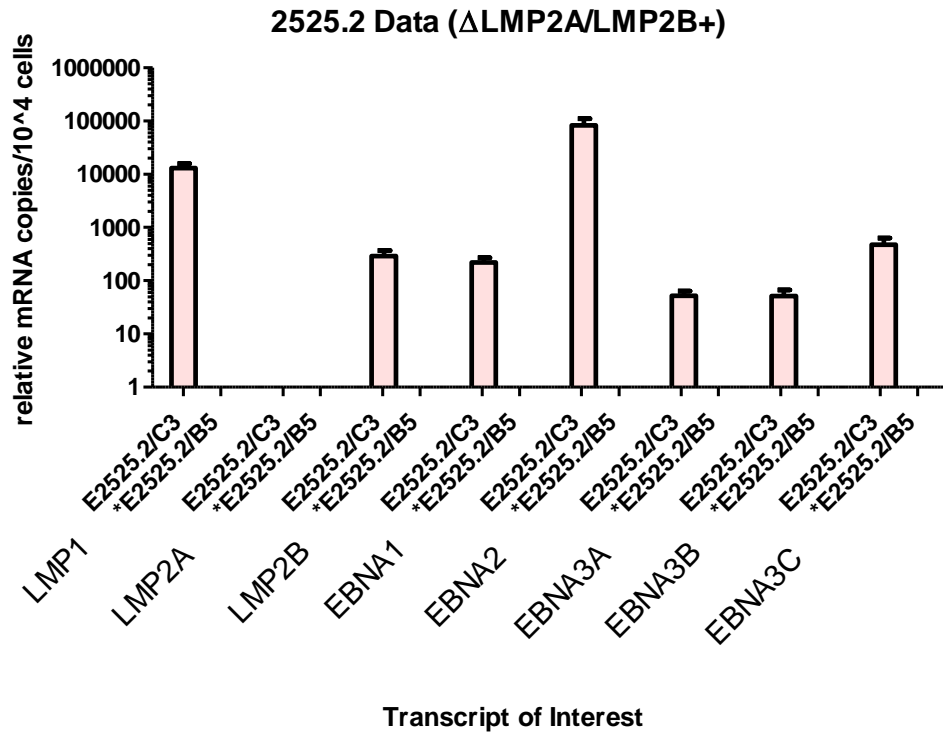


Figure 9. Gene transcription within the 2525.2 Cell Lines

Figure 9 depicts the difference between the 2525.2 producer cell line and the 2525.2 nonproducer cell line. Only two genes were actually detected for the 2525.2 nonproducer; these were the C2:W1 nonproductive splice and the W0:W1 nonproductive splice.

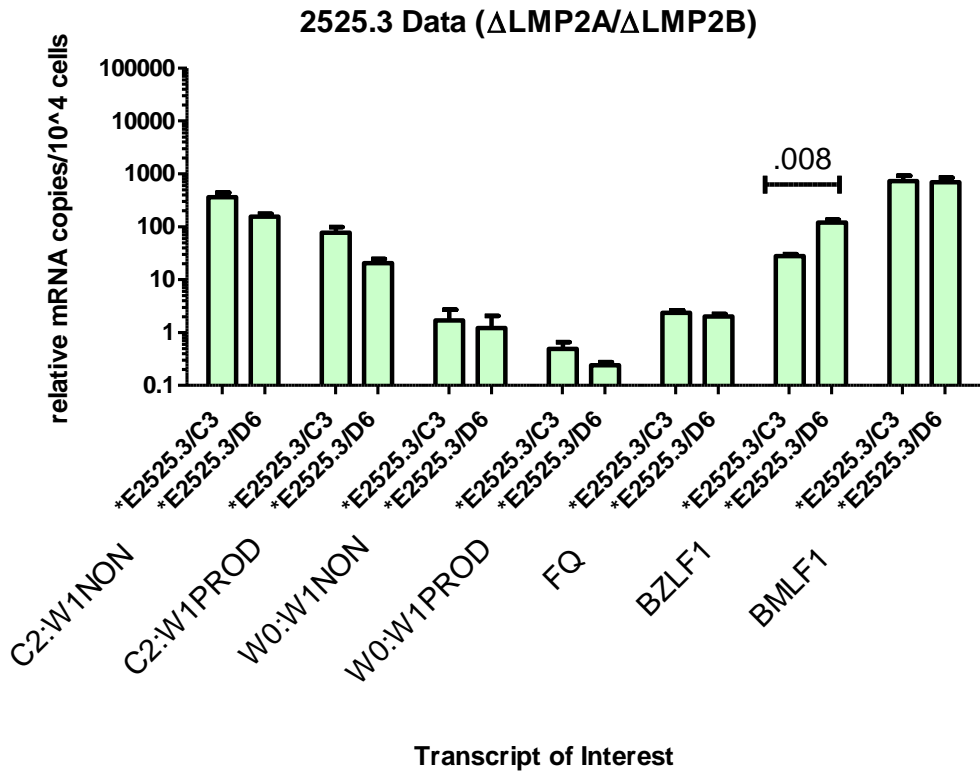
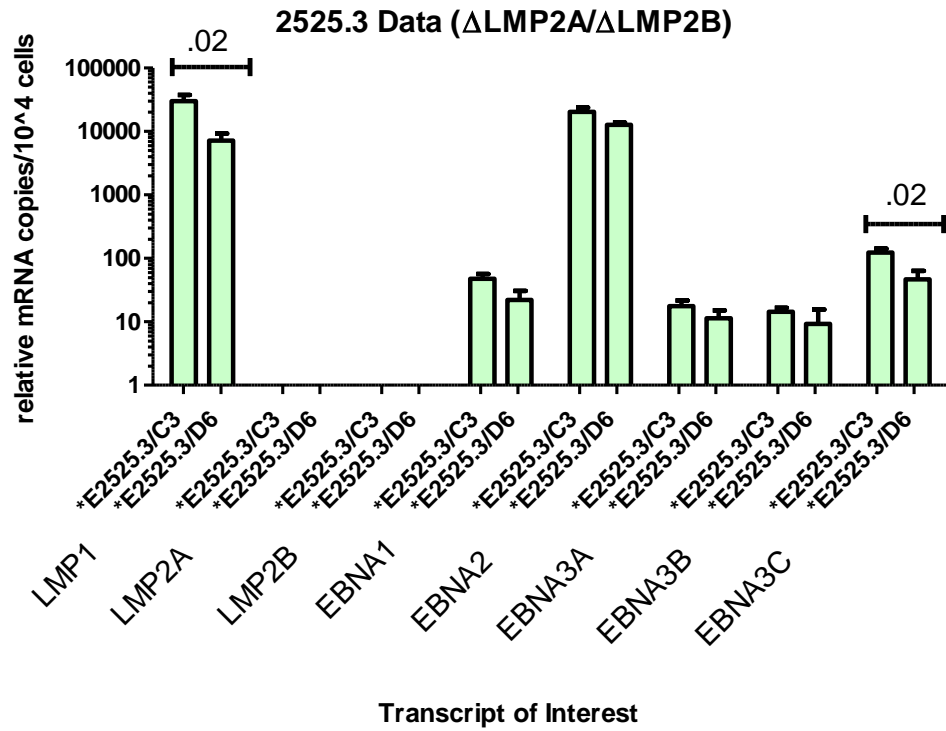


Figure 10. Gene transcription within the 2525.3 Cell Lines

Figure 10 depicts differences between 2 nonproducers within the 2525.3 cell line. The only statistically significant gene measurement was in BMLF1 ($p=0.008$). The LMP2B transcript was again missing as expected in the LMP2B LoxP deleted cell line.

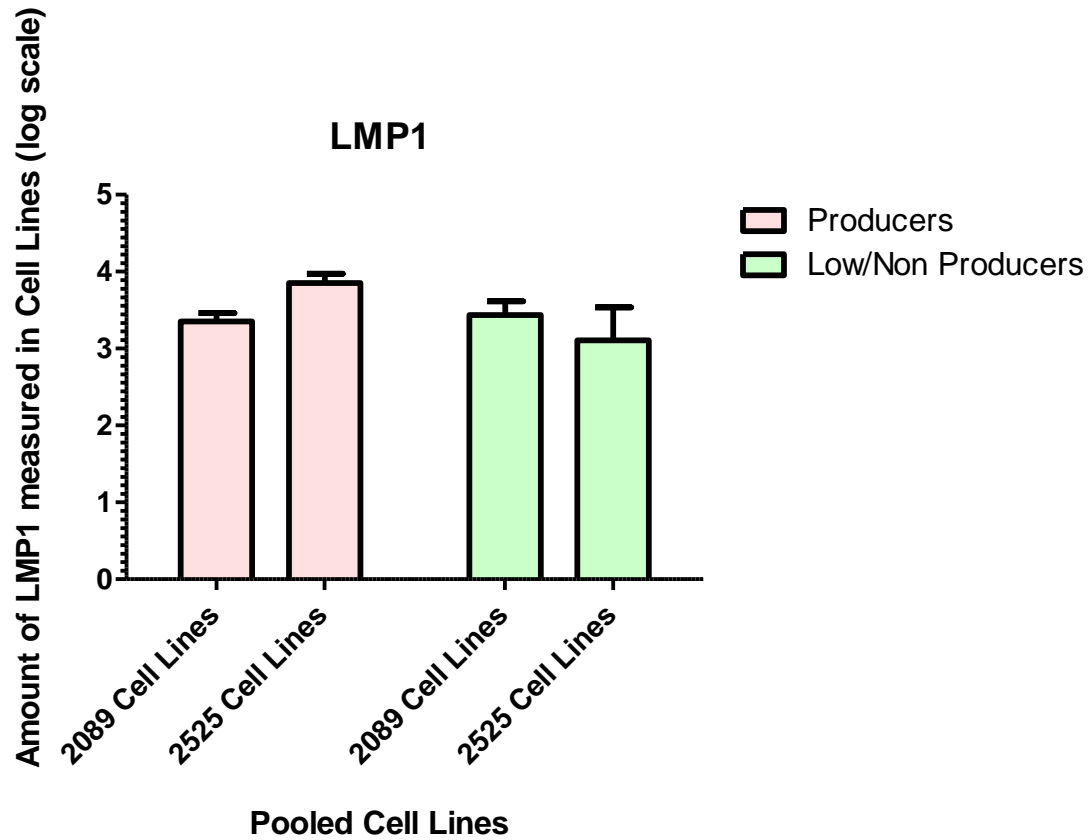


Figure 11. LMP1 analyzed with Two Way ANOVA

There was no significant difference in LMP1 expression between pooled producers, pooled low/nonproducers, pooled 2089 cell lines or pooled 2525 cell lines.

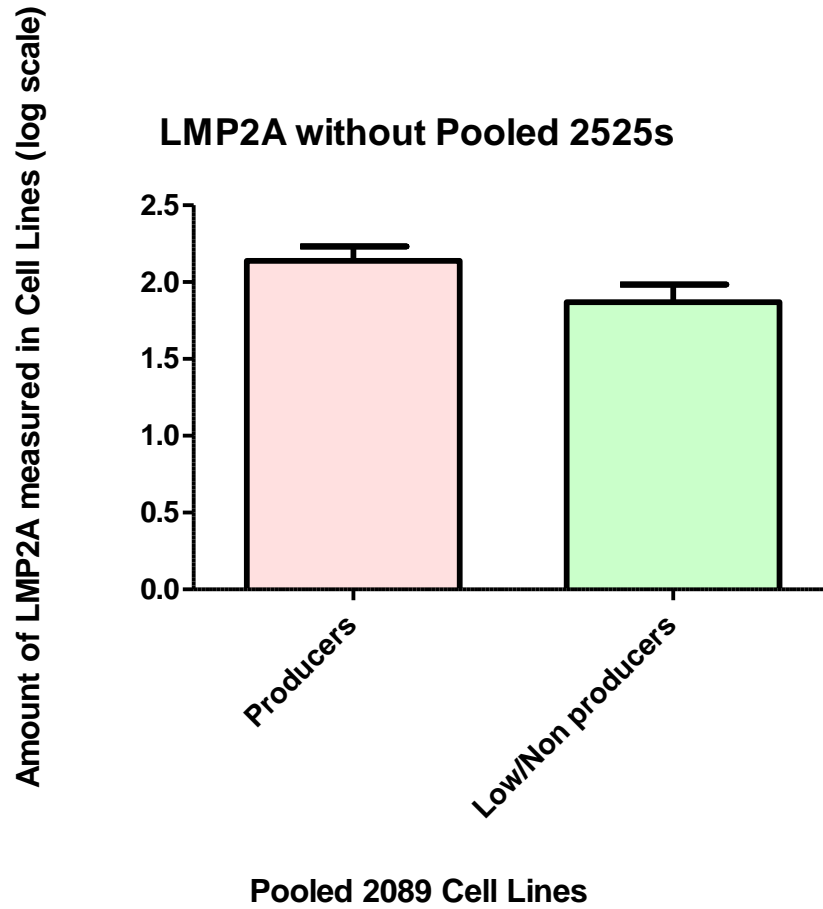


Figure 12. LMP2A analyzed with a two-tailed unpaired Student's t-test.

LMP2A expression was not significantly different between pooled producers and low/nonproducers. This figure shows only the pooled 2089 cell lines because the 2525 cell lines have a LoxP deletion and cannot express LMP2A.

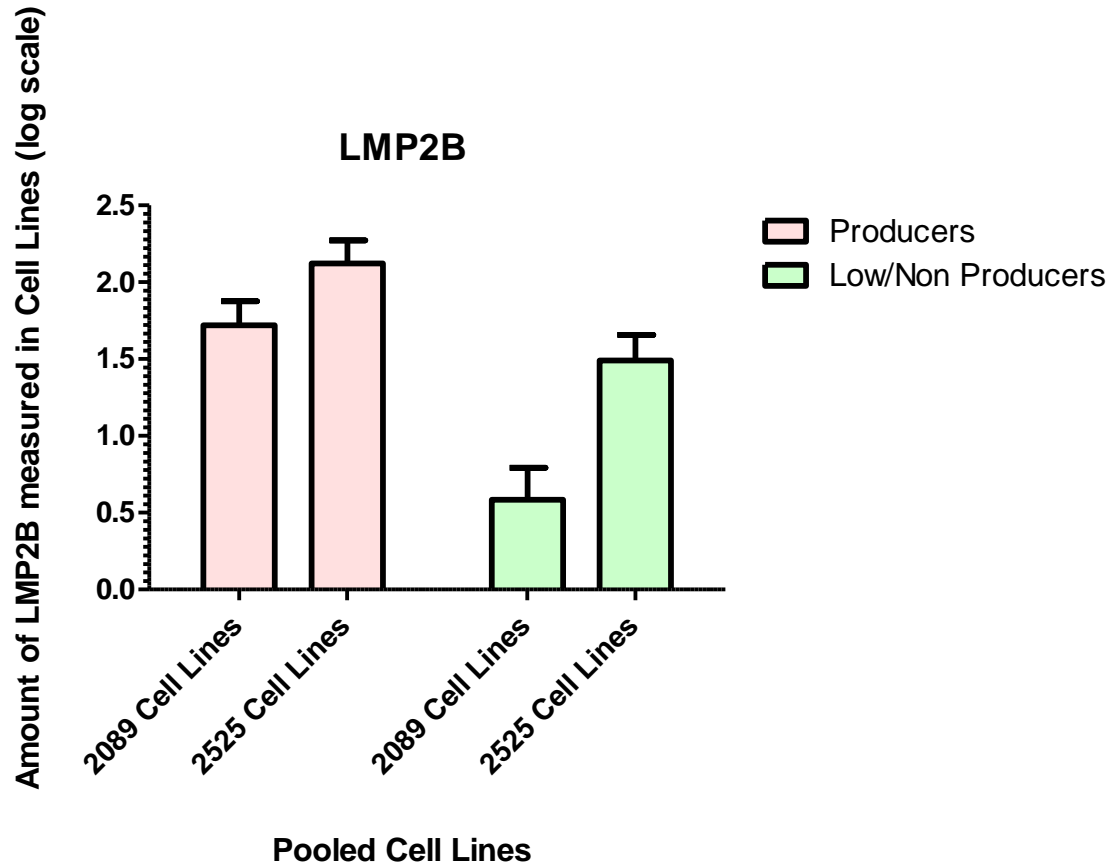


Figure 13. LMP2B analyzed with Two Way ANOVA

Figure 13 shows that the LMP2B expression of pooled producers was statistically significantly different from the low/nonproducers, $p=0.0004$. There was also a slight interaction between ANOVA comparisons suggesting that the pooled 2089 cell lines tended to produce slightly less LMP2B than the pooled 2525 cell lines regardless of producer or low/nonproducer status, $p=0.005$. The cell lines that do not express LMP2B because of a LoxP deletion (2089.3 and 2525.3) have not been included in this analysis.

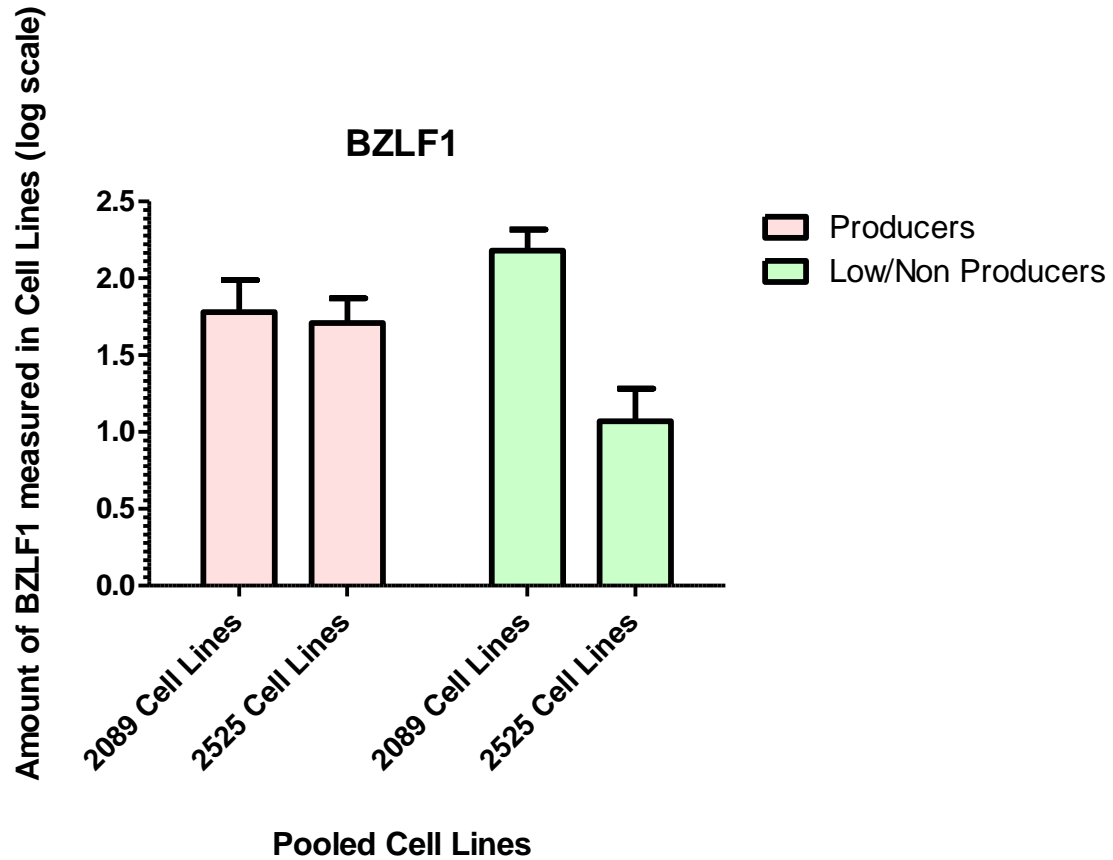


Figure 14. BZLF1 analyzed with Two Way ANOVA

In Figure 14 there was a very slight significant difference between pooled producers and pooled low/nonproducers, with the low/nonproducers making only slightly more BZLF1, $p=0.01$. BZLF1 did express differently though depending on which cell lines it was being produced in, $p=0.007$. In the pooled producers the 2089 and 2525 cell lines made similar amounts of BZLF1. In the pooled low/nonproducers the 2089 cell lines made more BZLF1 than the 2525 cell lines.

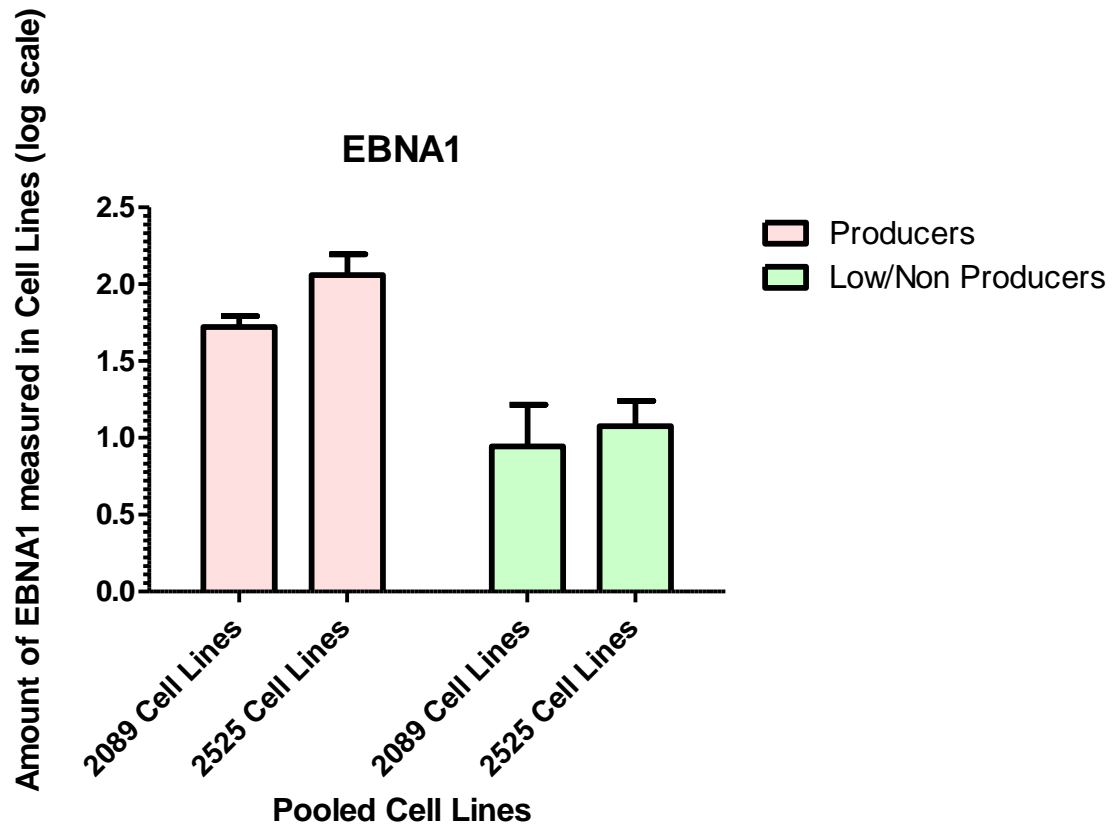


Figure 15. EBNA1 analyzed with Two Way ANOVA

Pooled producers made more EBNA1 than pooled low/nonproducers for both 2089 and 2525 cell lines, $p=0.0002$.

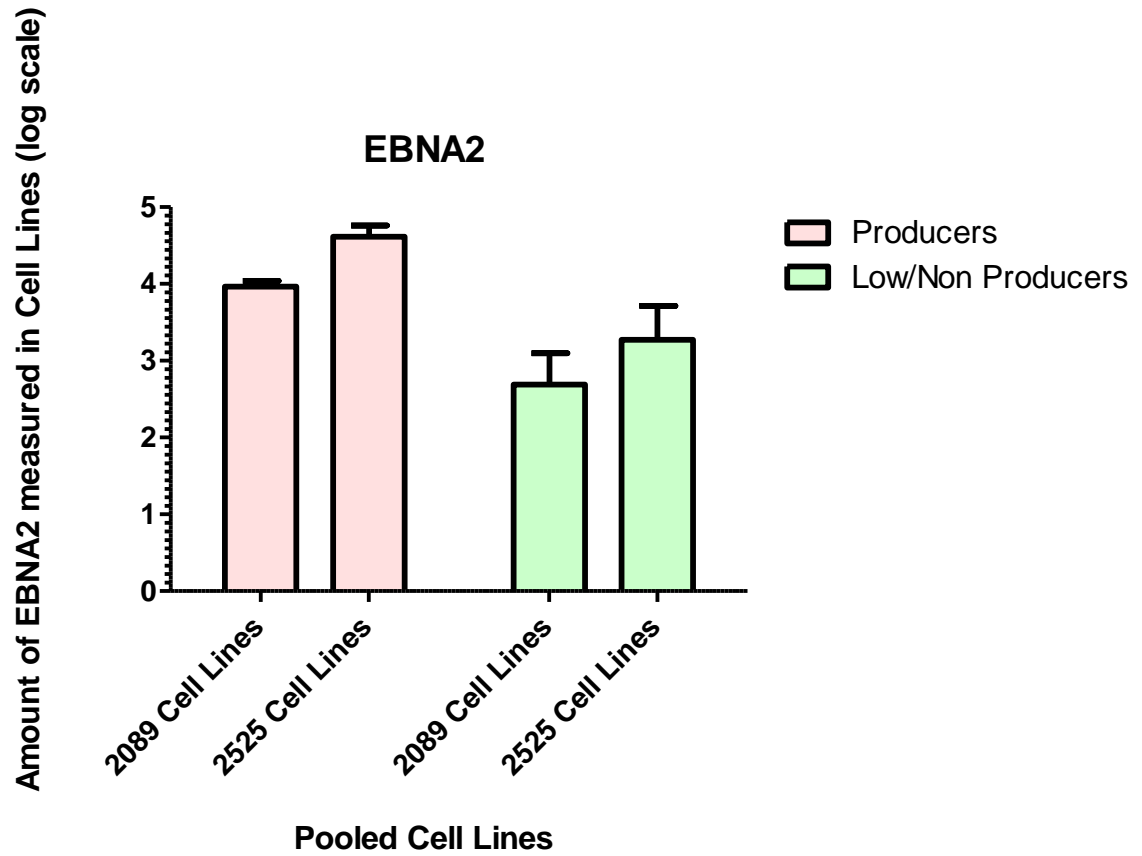


Figure 16. EBNA2 analyzed with Two Way ANOVA

Pooled producers made more EBNA2 than pooled low/nonproducers for both 2089 and 2525 cell lines, $p=0.0018$.

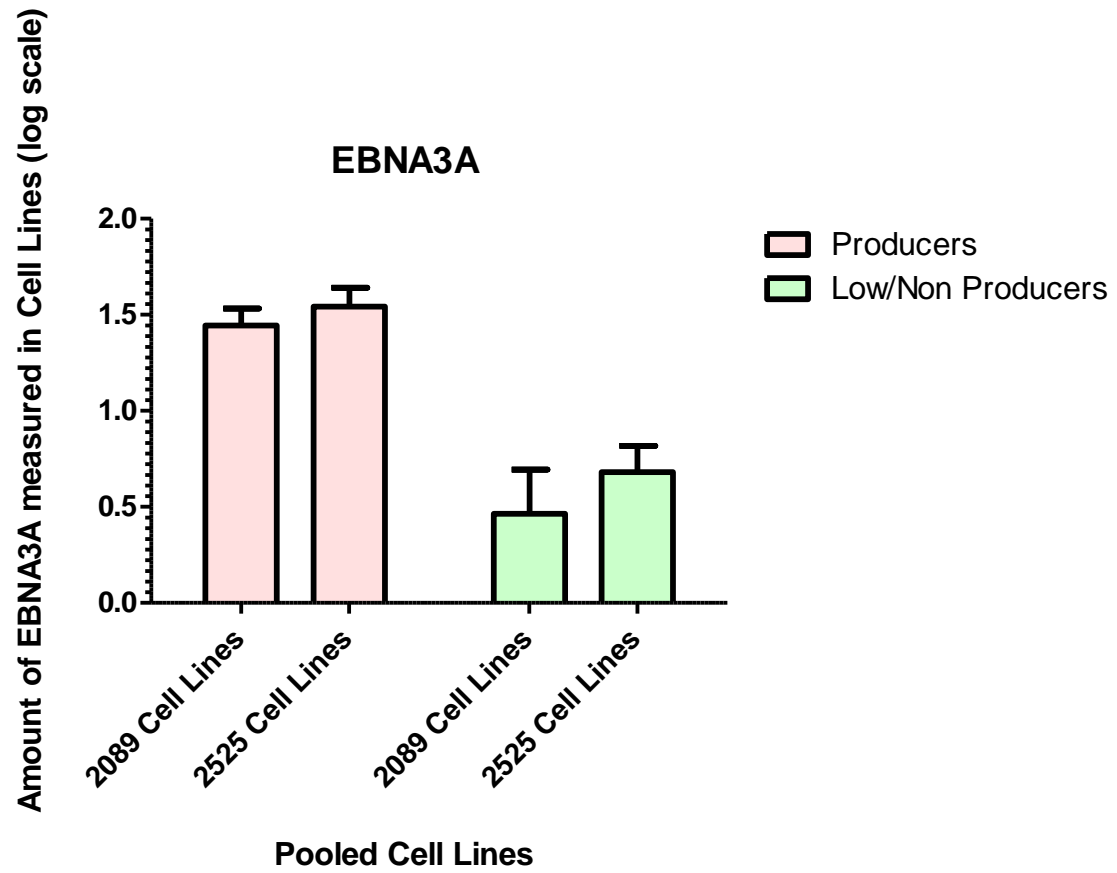


Figure 17. EBNA3A analyzed with Two Way ANOVA

Pooled producers made more EBNA3A than pooled low/nonproducers for both 2089 and 2525 cell lines, $p < 0.0001$.

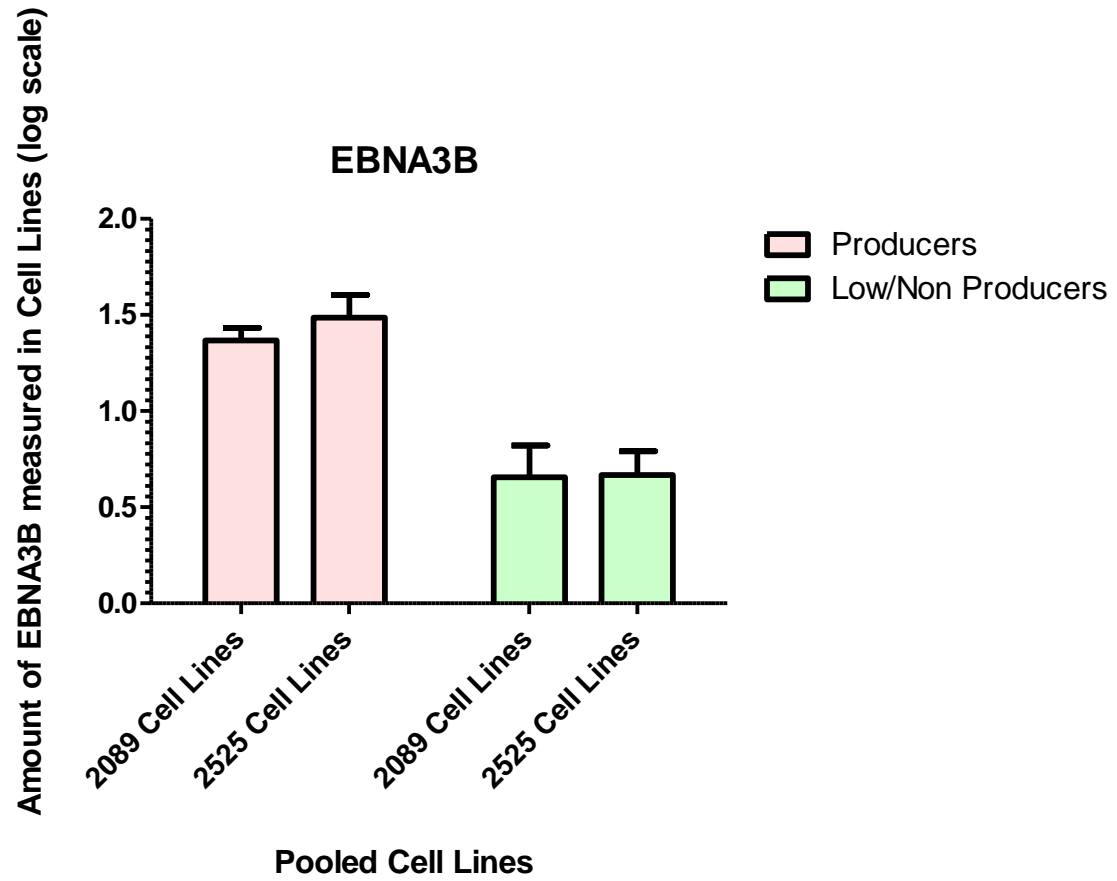


Figure 18. EBNA3B analyzed with Two Way ANOVA

Pooled producers made more EBNA3B than pooled low/nonproducers for both 2089 and 2525 cell lines, $p < 0.0001$.

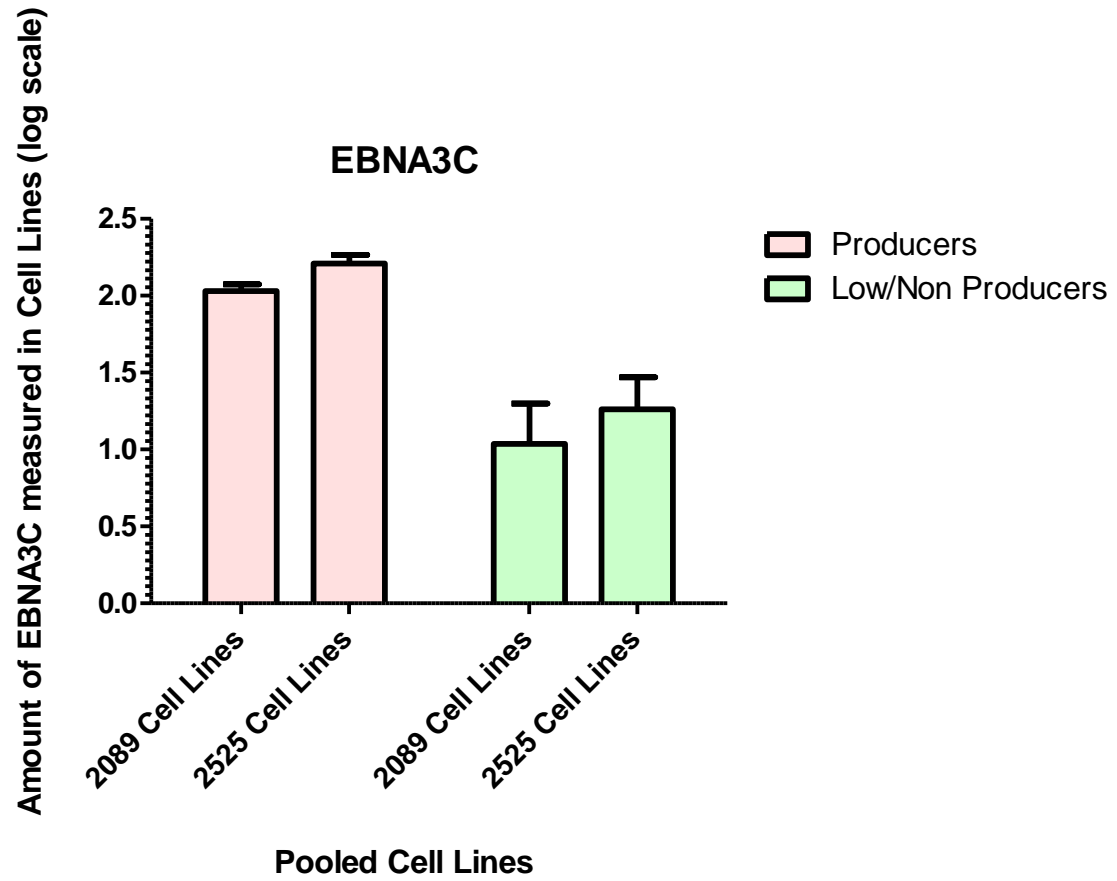


Figure 19. EBNA3C analyzed with Two Way ANOVA

Pooled producers made more EBNA3C than pooled low/nonproducers for both 2089 and 2525 cell lines, $p < 0.0001$.

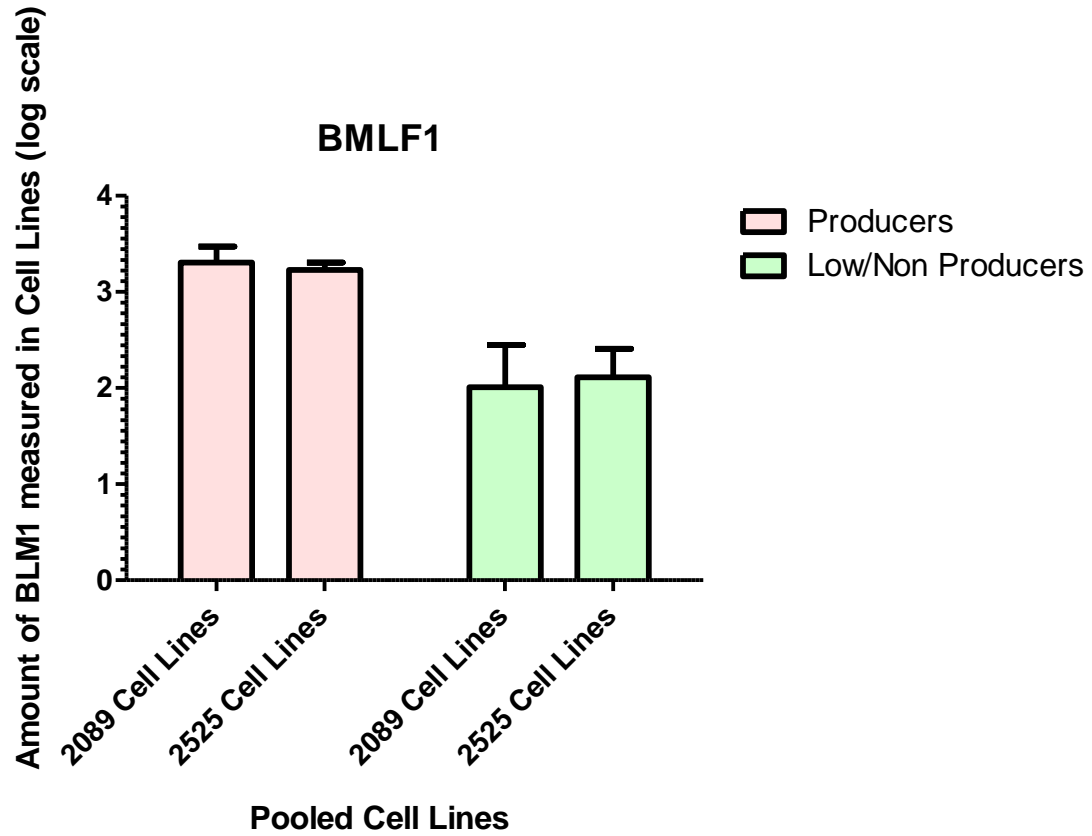


Figure 20. BMLF1 analyzed with Two Way ANOVA

Pooled producers made more BMLF1 than pooled low/nonproducers for both 2089 and 2525 cell lines, $p=0.0016$.

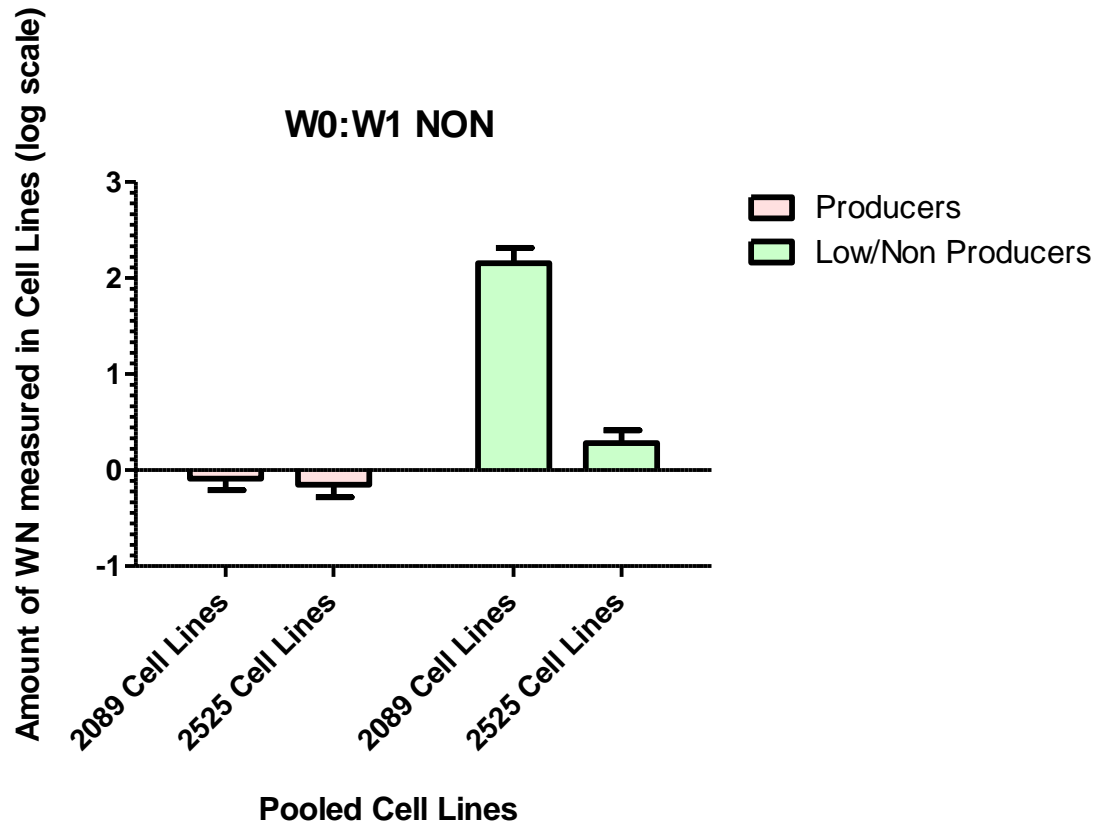


Figure 21. W0:W1 NON analyzed with Two Way ANOVA

The W0:W1 NON transcript was differently expressed depending on both producer status and which cell line was expressing it, $p < 0.0001$. The pooled producers made fewer transcripts than the pooled low/nonproducers. Within the pooled producers the 2089 cell lines made fewer transcripts than the 2525 cell lines. Within the pooled low/nonproducers the 2089 cell lines made more transcripts than the 2525 cell lines.

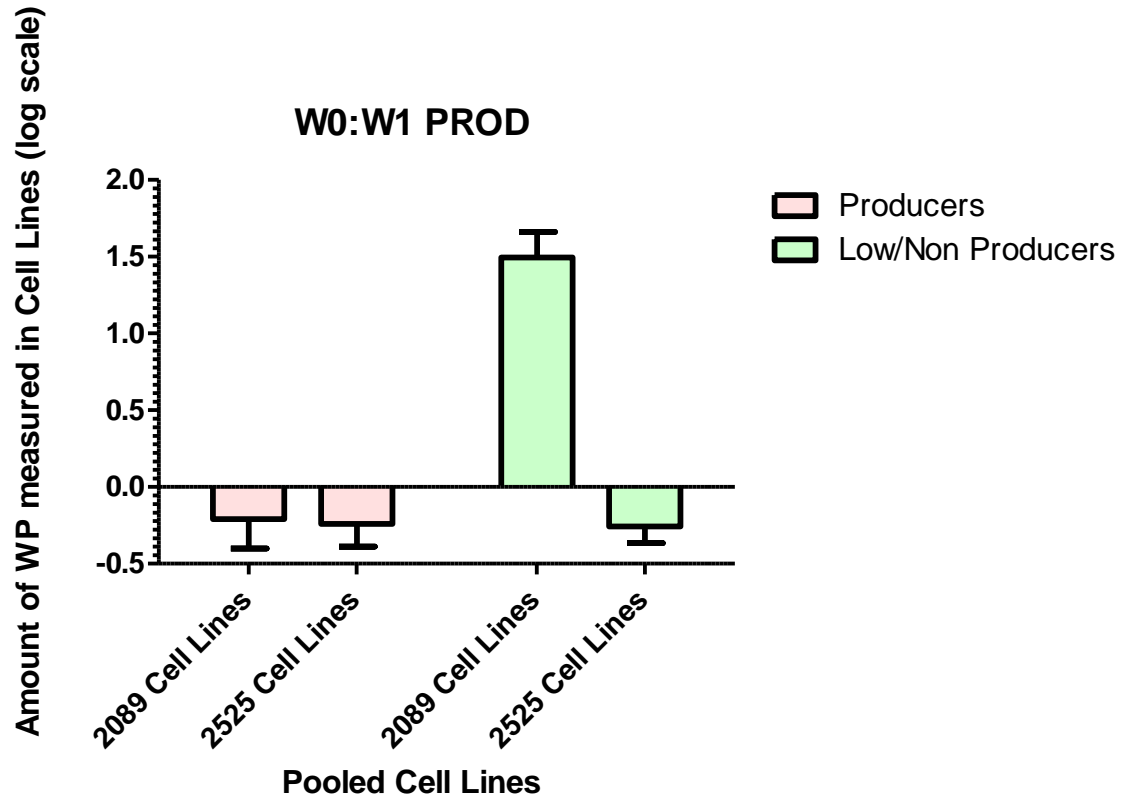


Figure 22. W0:W1 PROD analyzed with Two Way ANOVA

The W0:W1 PROD was differently expressed depending on both producer status and which cell line was expressing it, $p < 0.0001$. The pooled producers made fewer transcripts than the pooled low/nonproducers. Within the pooled producers the 2089 cell lines made fewer transcripts than the 2525 cell lines. Within the pooled low/nonproducers the 2089 cell lines made more transcripts than the 2525 cell lines.

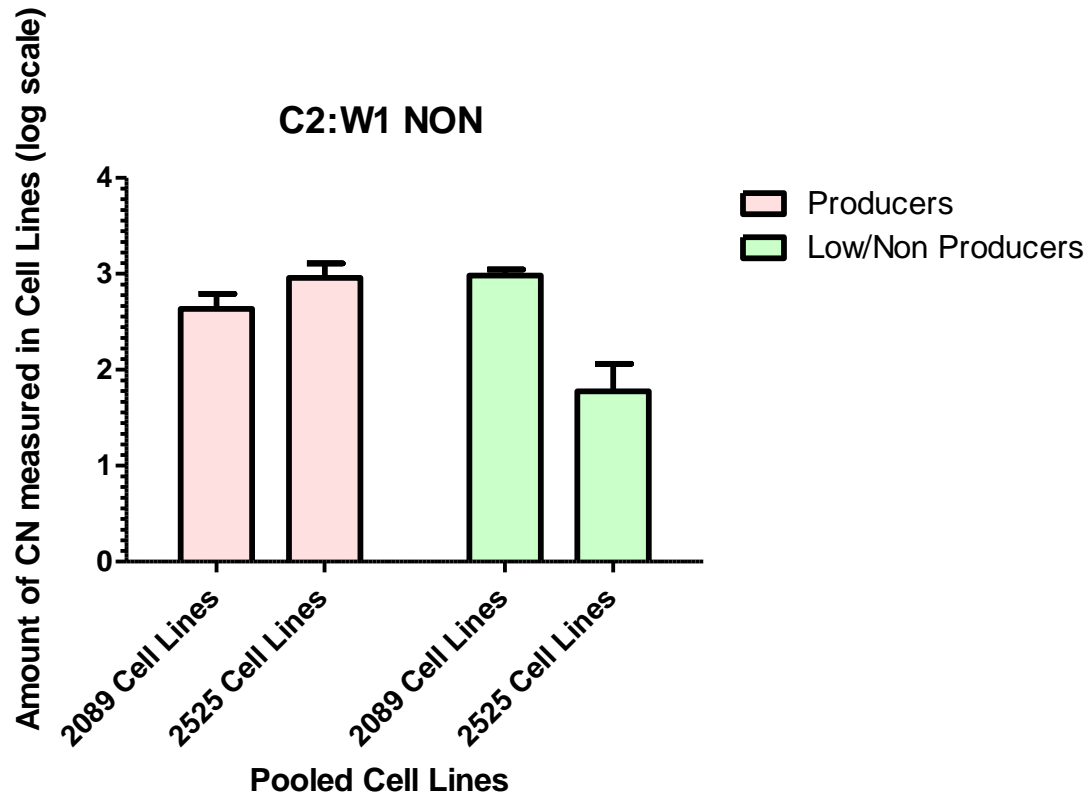


Figure 23. C2:W1 NON analyzed with Two Way ANOVA

The C2:W1 NON transcript was expressed differently depending on both producer status and which cell line was expressing it, $p=0.0009$. The pooled producers made a similar amount of transcript to the pooled low/nonproducers. Within the pooled producers the 2089 cell lines made fewer transcripts than the 2525 cell lines. Within the pooled low/nonproducers the 2089 cell lines made more transcripts than the 2525 cell lines.

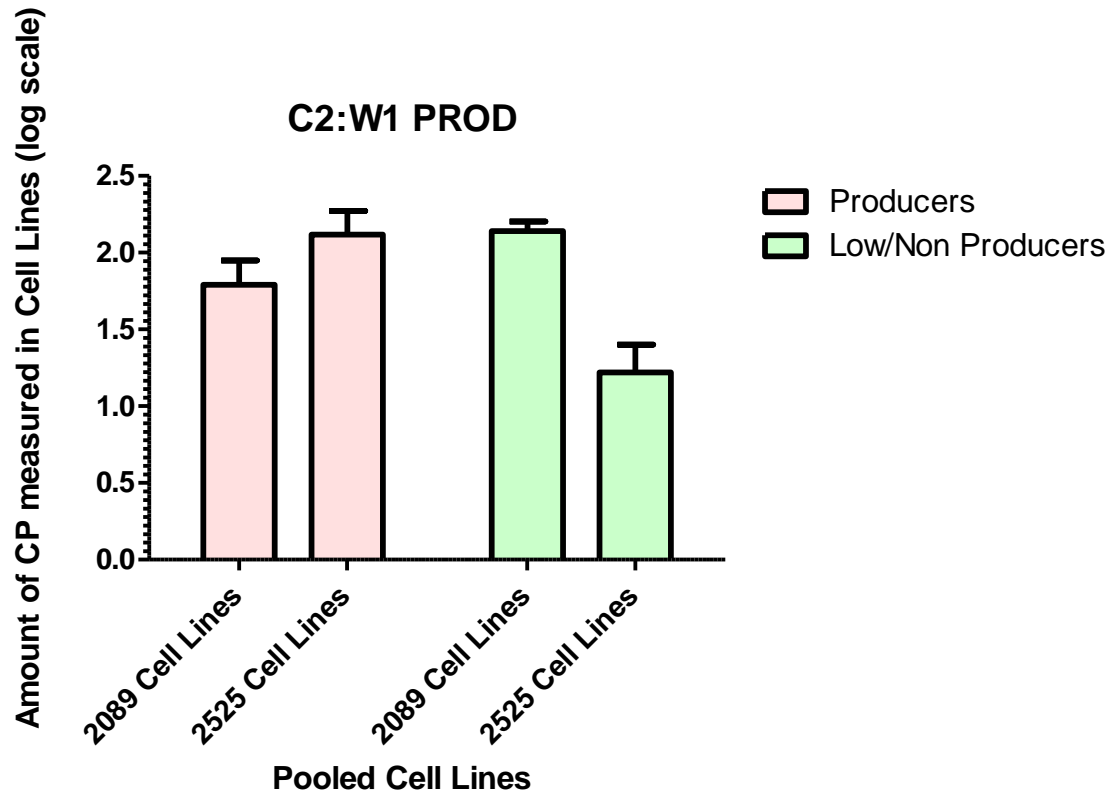


Figure 24. C2:W1 PROD analyzed with Two Way ANOVA

The C2:W1 PROD transcript was expressed differently depending on both producer status and which cell line was expressing it, $p=0.0004$. The pooled producers made a similar amount of transcript to the pooled low/nonproducers. Within the pooled producers the 2089 cell lines made fewer transcripts than the 2525 cell lines. Within the pooled low/nonproducers the 2089 cell lines made more transcript than the 2525 cell lines.

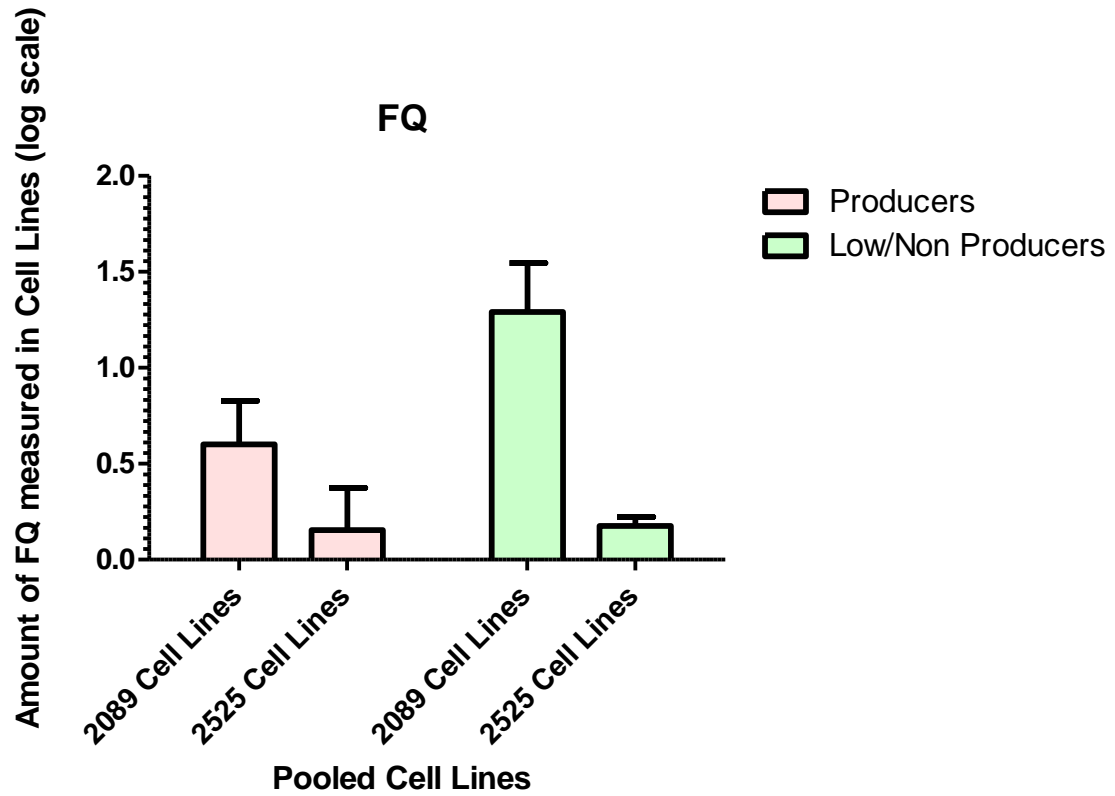


Figure 25. FQ analyzed with Two Way ANOVA

FQ promoter activity was only significantly different between pooled 2089 and 2525 cell lines, $p=0.001$, and was not significantly different between pooled producers and pooled low/nonproducers.

4.0 DISCUSSION

While a number of the differences detected in this study were not statistically significant within each cell line, we were primarily searching for a reasonably consistent difference between producers and low/nonproducers. There was no data obtained for the BamHI A rightward transcripts as this region has been deleted in the B95.8 virus strain used as the backbone for the BACs. As shown in Figures 4 through 10 we observed differences within each cell line, however there was too much variability in gene transcription to derive statistical significance between cell lines and to determine that there was an effect of latent EBV gene transcription on virus production. This makes it very difficult to determine whether these genes can be used as predictive indicators of producer status for future experimental screens.

Figures 11 through 25 pooled results by producer status and clarified the possibilities to some degree. The pooling of producers and low/nonproducers, as well as the pooling of 2089 and 2525 cell lines made it easier to view the overall picture of gene expression. Based on the ANOVA analyses we can group the results into not useful, somewhat useful, and potentially useful for determining predictive differences.

LMP1 (Figure 11), BZLF1 (Figure 14), LMP2A (Figure 12), and FQ (Figure 25) transcription would not be useful in determining producer status in untested cell lines in the future. LMP1 showed no difference in transcription under any condition. LMP2A showed no difference between pooled producers and pooled low/nonproducers. BZLF1 was different

depending on both 2089 and 2525 cell line grouping as well as both producer and low/nonproducer status. This made these genes not useful as indicators of virus production. FQ only showed differences between pooled 2089 and pooled 2525 cell lines. Though this observation may be interesting it is not useful in determining differences between producers and low/nonproducers.

The promoter proximal splice usages of W0:W1 NON (Figure 21), W0:W1 PROD (Figure 22), C2:W1 NON (Figure 23) and C2:W1 PROD (Figure 24) can be categorized as somewhat useful. In the W promoter regions it was clear that the producers always generated fewer transcripts than the low/nonproducers, even though there was also a difference in transcription between pooled 2089 and pooled 2525 cell lines. When comparing the C promoter region (Figures 23 and 24) with the W promoter region (Figures 21 and 22), the C promoter regions appeared to be producing more transcripts than the W promoter regions. This could possibly be a pattern to look for as a predictive difference. This pattern may be related to the gradual replacement of W promoter function by C promoter function in LCLs during the establishment of latency III in B cells (6), even though this study used transfected 293 cells and not B cells. What does not seem to correlate is the total amount of EBNA transcripts (3' ends) detected and the amount of combined C and W promoter proximal (5' ends) transcripts being made. This may suggest that some other promoter is functioning in the transfected 293SL cells to make the EBNA transcripts, or perhaps that the downstream coding regions of mRNA are more stable than the upstream promoter regions of the mRNA.

The genes that appear to be most useful for possibly predicting a difference between future producers and low/nonproducers would be EBNA1 (Figure 15), EBNA2 (Figure 16), EBNA3A (Figure 17), EBNA3B (Figure 18), EBNA3C (Figure 19), BMLF1 (Figure 20), and

LMP2B (Figure 13). All of the pooled producers for these genes generated significantly more transcripts than the pooled low/nonproducers regardless to which pooled cell line the producers belonged. These genes would be the ones to look at in a PCR assay designed to predict possible producer status. One possible exception is LMP2B. While LMP2B does show a significant difference between producers and low/nonproducers it is not present in all cell lines (e.g., 2089.3 and 2525.3).

5.0 CONCLUSIONS

The genes most useful for creating a PCR assay based on viral gene expression that could possibly predict which transfected 293 cell lines would be producers would be the genes belonging to the EBNA3 family. The EBNA3 expression in pooled producers was more significantly different than any of the other genes that could possibly be used to predict virus production status (EBNA1, EBNA2, and BMLF1).

If a PCR assay was designed using the EBNA genes, then tested and shown to accurately predict producer cell line status, this would reduce the effort, time, and cost currently being invested to determine whether cell lines are virus producers and greatly increase the chances that a useful producer is among the cell lines being expanded for testing. The public health significance of the efficient production of Epstein-Barr virus recombinants would be that experiments on the viral functions associated with virus transformation of B cells (the key to the pathogenesis of EBV related diseases) could be conducted with greater numbers of variants on shorter time tables, which in turn may increase the rate of knowledge obtained about EBV.

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