Norwegian University
of Life Sciences

Master's Thesis 2022 30 ECTS
Faculty of Biosciences

Developing Cas9 expressing MDBK cell lines for GeCKO screening against bovine diseases

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Acknowledgement

I would like to express my sincere gratitude towards my supervisors Thomas Nelson Harvey and Victor Boyartchuk. Right from the experimental design they helped me a lot in understanding and contemplating science. Thank you for replying to my mails and doubts even during late hours. I would also be indebted to Matthew Peter Kent for involving me in attending the Geneinnovate meetings and encouraged me to excel in all the ventures I undertook. I also thank my fellow lab mates and laboratory technicians.

Abstract

Cas9 expressing MDBK (Madin-Darby bovine kidney) cell lines were produced for a GeCKO (genome scale CRISPR/Cas9 gene knockout) screening against Bovine diseases. The lines were transduced with lentiviral vectors containing Cas9 and quantified for RNA expression and protein levels. sgRNAs were designed and assembled into lentiviral vectors. Transduction of Cas9 expressing MDBK cell lines with sgRNA containing lentivirus resulted in CRISPR/Cas9 gene editing. Editing efficiencies of selected lines were compared and correlated with their Cas9 expression profiles.

Content

1. Introduction

Rise in global population, over consumption and environmental degradation has always exerted negative influence on food systems (Herrero & Thornton, 2013). High density farming of livestock in confinement using modern breeding techniques was a practical solution put forward, but raising a large population of animals in close proximity induced possible chances of disease outbreaks (Otte et al., n.d.). The development of effective methods for preventing these diseases was a necessity and could positively impact farm welfare, global trade and human health (Hennessy & Wolf, 2018).

Viral diseases are one of the major infectious diseases that affect livestock and there has been a steady spike in its emergence in the last few decades. Among the viral diseases, bovine viral diarrhea (BVD) and bovine corona virus (BCoV) infection are two of the recurrent single stranded RNA virus causing diseases infecting calf intestinal and respiratory tracts (Storz et al., 2000).

Animals infected with BVD develop acute diarrhea which significantly affects the production. Also during the recovery, the immune system is compromised which increases the susceptibility for secondary infection (*Bovine Viral Diarrhea*, 2019). BVD instigate much more complicated clinical presentations involving newborn stillbirths, teratogenic malformations, abortions, and parturition of immunotolerant animals (Gomez-Romero et al., 2021). Viral species associated with BVD belongs to genus Pestivirus under family Flaviviridae and the infectious genotypes include bovine viral diarrhea virus 1 (Pestivirus A), Bovine viral diarrhea virus 2 (Pestivirus B), and Hobi-like pestivirus (Pestivirus) (Gomez-Romero et al., 2021; Smith et al., 2017).

Clinical presentations of BCoV infection include respiratory infections-often associated with bovine respiratory disease complex (BRDC), neonatal calf diarrhea, and winter dysentery. They belong to the betacoronavirus (mammalian coronavirus) genus under coronaviridae family and the infectious genotype is a single pneumocentric betacoronavirus 1 species, having different host specificities (Vlasova & Saif, 2021).

Studies using interference of monoclonal antibodies (MAbs) in bovine cells and resulting cytopathogenicity of BVDV (Teyssedou et al., 1987) suggested the possible involvement of surface receptor mediated entry of virus. Subsequent studies elucidated the participation of several cellular receptors including several glycosaminoglycans (GAGs) (Hulst et al., 2001) low-density

lipoprotein receptor (LDL-R), and prominently bovine membrane cofactor protein CD46 (Maurer et al., 2004, p. 4).

Even though quite a few scientific publications could be retrieved regarding the targeted surface receptors involved in host-pathogen interactions of BVDV, there are very less studies that holistically give an account of the multitude of translated cellular receptor protein genes. Correspondingly, there are some studies indicating the effect of N-acetyl-9-O-acetylneuraminic acid as a receptor determinant in BCoV infection initiation (Schultze & Herrler, 1992) but not much is known about the receptors itself. This lacuna present in the current research opens the possibility for initiating studies, predominantly involving powerful ways to identify novel proteins and their functions associated with viral recognition and progression.

CRISPR based screening of cells lacking a defined gene/genes in a genome scale could be used as robust tool in identifying candidate cell surface receptor genes (Sharma & Wright, 2020). The potentiality of programming CRISPR/Cas9 to specifically target a DNA sequence using short guide RNAs revolutionized the existing field of genome wide screening (Knott & Doudna, 2018). There are different combinations of CRISPR screening modalities including survival screening and fluorescence-activated cell sorting (FACS), survival and arrayed, arrayed and FACS, arrayed and single-cell or FACS and single cell (Shalem et al., 2014).

Selection of the appropriate screen depends upon the phenotype exhibited by the cell and the scale of experiment. Since the infection of cytopathic (CP) biotype of BVDV induce autophagy and solely dependent on the viral infection and progression (Zhang et al., 1996), it can be used as a dominant phenotype. A genome scale CRISPR/Cas9 gene knock out (GeCKO) screen could be used in the experimental set up where a CRISPR cell line is transfected with pooled sgRNA library targeting all protein coding genes. The genes could be identified from the cells exhibiting survival phenotype by deep sequencing, whose knockout conferred strong protection against BVDV induced cell death.

The major cell lines used for screening against BVDV and BCoV is Madin-Darby Bovine Kidney cells (MDBK). It is regarded as an acceptable model cell line as a transfection host in bovine diseases (*MDBK (NBL-1)* | *ATCC*, n.d.) and numerous previous research works uses it as a successful model in finding effects of livestock viral infection (Cristina et al., 2001; Fay et al., 2020; Tark et al., 2015).

Original research in optimizing GeCKO libraries (Sanjana et al., 2014; Shalem et al., 2014) suggests different methods of developing lentiviral CRISPR plasmids targeting single genomic locus. The reason for selecting lentiGuide-Puro (two vector system) over lentiCRISPRv2 (one vector system) is its higher relative functional viral titer (Sanjana et al., 2014) and also invokes the possibility for multiple screening experiments. Since the efficiency of GeCKO screening also depends on the knockout efficiency (Supplemental figure, Sanjana et al., 2014), particular care must be provided in selecting the Cas9 expressing cell lines in case of the two-vector system. The scope of this thesis resides in two major objectives:

1. Developing and selecting Cas9 expressing MDBK cell lines for subsequent screening.
2. Correlate the editing efficiency of the lines based on their Cas9 levels.

2. Materials and Methods

2.1.Cell culture

Madin-Darby bovine kidney (MDBK) cell lines and HEK 293T cell lines were cultured Dulbecco's Modified Eagle's Medium - high glucose (DMEM) (D6429-500mL) 10% Fetal Bovine Serum (FBS) gold (FCS.GP.0500) was added as supplement (instead of FBS to eliminate possible chances of interference during experimental results due to contamination with Bovine Viral Diarrhea Virus) with 1% of antibiotic Pen-Strep (mixture of penicillin G and streptomycin, Thermo 15140122) and L-glutamine (2mM, Thermo 25030024).

Cryopreserved Madin-Darby bovine kidney cell lines (NBL-1, Adherent cell type) from NTNU (TCC, lot tested negative for BVDV) was used as the permanent cell line for most of the experiments. The lines were thawed quickly and transferred to 10 ml of the standard growth media and spun down by centrifugation (200g for 5 min) and media discarded. The cells were then resuspended in fresh media by pipetting. The lines were grown in vented T75 EasYFlask in CO2 incubator at 37°C and 5% CO2. The cells were maintained for a period of 5 months by periodic change of media and constant subculturing (34 passages). HEK 293T cell lines were used for two lentivirus production experiments (due to the presence of SV40 T-antigen which promotes cellular transformation in the lines). Same thawing method for MDBK lines were used. Standard DMEM growth media (with FBS, gold) was used for cell culture.

Both the lines (MDBK and HEK-293T) were incubated at the same temperature, CO2 conditions (37°C, 5% CO2) unless direct working with it. Biosafety cabinets were used for any process that involved cell lines. MDBK cell lines obtained were robust and reached 80-90% confluent in 48 hours if seeded with 2x10^5 cells. The adherent cells were washed with 2mL of 0.25% trypsin (Thermo 2520072) and either kept in culture conditions (37°C, 5% CO2) for 5 min or 15 min in room temperature. The cells were then washed using media and counted using Bio-Rad TC20 automated cell counter and seeded in T75 flasks.

Dulbecco's Phosphate buffered saline (PBS) (14200-067 500mL) without calcium or magnesium was used to wash HEK 293T cell lines prior to passaging. 2 mL of 0.05% trypsin (in PBS solution) was added to detach the cells. 8 mL of standard media was then added to make a total volume of 10 mL. Cells were then counted using TC20 automated cell counter and seeded in T75 flask (2 x

10^6 cells in 10 mL final volume). HEK 293 cells were passaged after 48 hours, and the media is changed in between if a pH change is observed.

2.2.Lentivirus packaging

HEK 293T cell lines, after two passages were used for LentiCas9 production. Optimized protocol from Broad institute GPP Web portal was used 'shRNA/sgRNA/ORF Low Throughput Viral Production (10cm dish/6 well)'(*GPP Web Portal - Protocols*, n.d.). 2.2 x 10^5 cells were seeded in a single well of a 6-well plate using standard growth media and incubated for 24 hours (37°C, 5% CO2).

Addgene safety packaging and envelope plasmids were used for transfection. psPAX2 (psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260 ; http://n2t.net/addgene:12260 ; RRID:Addgene_12260)) was used as the packaging plasmid and pMD2.G (pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259 ; http://n2t.net/addgene:12259 ; RRID:Addgene_12259) / pVSV-G (pVSV-G was a gift from Akitsu Hotta (Addgene plasmid # 138479 ; http://n2t.net/addgene:138479 ; RRID:Addgene_138479), (Gee et al., 2020)) were used as envelope plasmids. lentiCas9-Blast (lentiCas9-Blast was a gift from Feng Zhang (Addgene viral prep # 52962-LV; http://n2t.net/addgene:52962; RRID: Addgene_52962)) and AAV-CMV-GFP (AAV-CMV-GFP was a gift from Connie Cepko (Addgene plasmid # 67634; http://n2t.net/addgene:67634; RRID: Addgene_67634)) were used as transfer plasmids. The experimental set up is shown in **Figure 1**.

The plasmid mixture was made in microcentrifuge tubes in the following ratio:

Reagent	**per well**
psPAX2	500 ng
pMD2.G/pVSV-G	50 ng
lentiCas9-Blast/CMV-GFP	500 ng
OPTI-MEM to total volume	37.5 uL

Table 1: Plasmid mixture for transfection

TransIT-LT1 (MIR 2300) was the transfection reagent used and was added dropwise to OPTI-MEM for dilution, mixed gently by pipette tip. 3ul of TransIT-LT1 and 15 uL of OPTI-MEM was prepared for each well. After 5 minutes of incubation in room temperature, transfection reagent was added dropwise to the plasmid mixture and mixed up by flicking the tubes. Incubation of the

transfection reagent-plasmid mix was done in room temperature for another 30 minutes. The transfection mix was then gently transferred to the HEK cells seeded in the 6 well plate and incubated until next morning. The media was then removed and replaced with 2.5 mL of fresh standard media and incubated for another 24 hours. After a day, the media containing lentivirus was harvested and preserved in a cryogenic vial in -80°C.

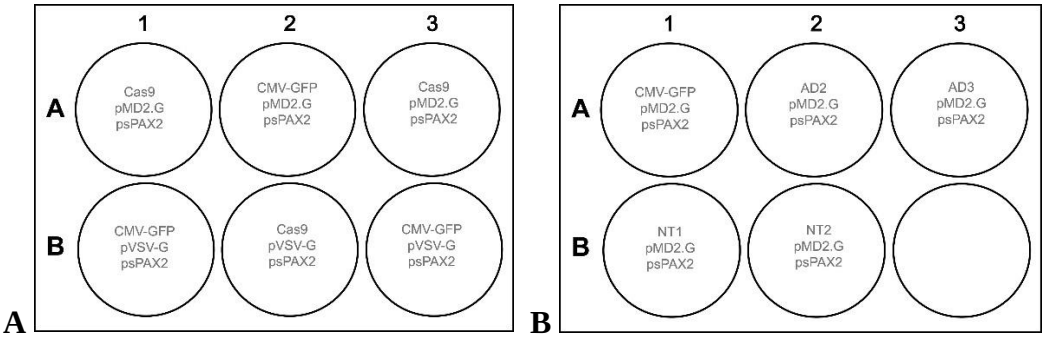


Figure 1: **A**: Transfer and envelope plasmid mix set up used for the first packaging experiment using lentiCas9Blast. **B**: plasmid mix set up for the second packaging experiment using lentiGuide-Puro

Second packaging experiment for plasmids with sgRNAs included a slightly different protocol in which lipofectamine 2000 (11668-019) was used as the transfection reagent. Also, the seeding density per well was different (7 X10^5 cells).

The plasmid mixture for this transfection experiment was in the following ratio:

Reagent	**per well**
psPAX2	750 ng
pMD2.G	500 ng
lentiGuide-Puro/CMV-GFP	1000 ng
OPTI-MEM	250 uL

Table 2: Plasmid mixture for transfection

The transfection mixture was prepared from 10 uL of lipofectamine 2000 and 250 uL of OptiMEM. Excluding these reagents and quantity, every step followed were similar to the first packaging experiment. The transfer plasmids that were used to package included lentiGuide-Puro backbone and inserts ADAM10_2 (AD2), ADAM 1_ 3 (AD3), BT104nt1 (NT1) and BT104nt2 (NT2) which were isolated from the positive colonies.

2.3. Transduction and selection for stable polyclonal MDBK lines

For transduction and antibiotic selection, optimized addgene protocol 'Generating Stable Cell Lines with Lentivirus' (*Addgene: Virus Protocol - Generating Stable Cell Lines*, n.d.) was used.

Blasticidin (ant-bl-05) was the selection antibiotic used as the lentiCas9Blast conferred specific resistance to the transfected cells. Before beginning the experiment, optimal dose for blasticidin was determined by treating MDBK cells with a range of doses (1ug-10ug/mL). The lowest dose that killed all the cells were determined. It was determined as 8ug/mL.

The lentiCas9Blast aliquots was rapidly thawed at 37°C and a range of dilutions was prepared using standard media and 10 ug/mL polybrene (TR-1003-G). Different dilution factors (ranged from 0, 1:5, 1:10, 1:50, 1:100) was made in each well of a 6-well plate to determine the optimum concentration of virus that could lead to successful selection. 0.5 mL of virus (virus titer unknown) + polybrene media (10 ug/mL) was added to each well, including a no virus control. A reverse transduction was performed by seeding 5 x 10^4 MDBK cells (in 2 mL media) to each well with virus (and mock infected) and incubated for 48 hours in 37°C, 5% CO2 incubator.

After 2 days, the media was gently aspirated and fresh media containing blasticidin was added. Each well was observed for any CPE effect or cell death. Regular change of media was done using same antibiotic concentration.

The experiment was repeated with higher virus concentration (300 uL) because the virus titer in the first experiment was too low. lentiCas9 blast produced from two different envelope plasmids (pMD2.G and pVSV-G) was used to determine which gave better stable lines. CMV-GFP lentivirus was also included in transduction to check the efficiency. After a week, stable cell lines expressing blasticidin resistance genes were obtained and was expanded to T25 flasks and subsequently to T75 flasks. These stable lines were then used to generate monoclonal lines.

2.4. Generating monoclonal cell population by serial dilution

Optimized Corning protocol 'Cell cloning by serial dilution in 96 well plates' (Ryan, n.d.) was used for monoclonal cell culture. Stable cell lines obtained from blasticidin selection was diluted as shown in the **figure 2**. Standard media is pipetted in equal amounts to all the wells except the first one using an 8-channel micropipette. Cells were suspended in standard media (2 x 10^4 cells/mL) and 200 uL from the cell suspension was added to the first well and diluted vertically

downwards using a micropipette. 1:2 dilution using 100 uL of cell suspension was done serially until it reached the last well and discarded the last 100 uL cell solution. Additional 100 uL of cell media was added to the first column to make the final concentration of 200 uL. Second dilution series was done horizontally using an 8-channel micropipette all the way from first column to the last one using the same tip (1:2 dilution). The volume of cell suspension is then made to a uniform 200 uL by adding 100 uL to all the wells. The experiment was done in four 96 well plates using all the blasticidin selected lines and were kept in 37°C, 5% CO2 for constant monitoring.

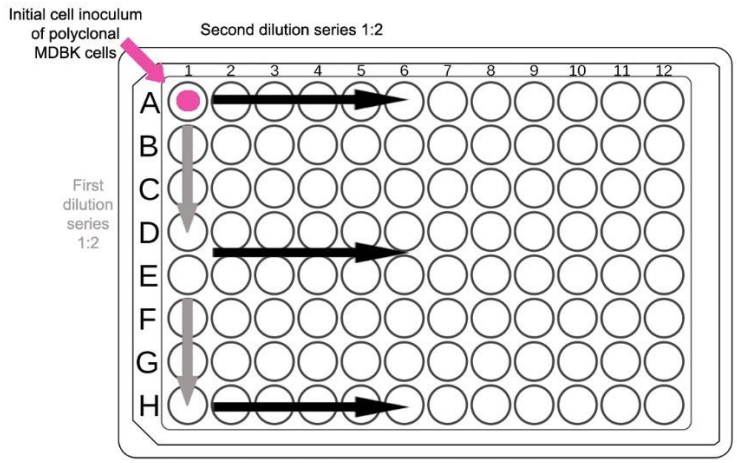


Figure 2: Experimental set up for single cell clones using serial dilution

Single cell clones were obtained from wells in lower right part of the plate in 5 days. Clones were identifiable in 9 days and the experiment continued until 2 complete weeks before expanding. Wells with more than one clones were discarded and 21 single cell clones were selected and expanded to 24-well plates, six well plates and then to T75 flasks. A sample from each line was then stored at -150°C in 10% DMSO (108K01864) for future use.

2.5. RNA isolation and qPCR

The cells were scrapped from the plates, washed with PBS and immediately kept on ice. A part was saved for western blotting and the other was used for RNA isolation. Total RNA was extracted using QIAGEN RNeasy Plus micro kit and stored in -20 °C. For the qPCR, iTaq Universal SYBR Green One-step Kit (172-5150) was used. The real time PCR system used was Bio-rad CFX96 in SYBR setting. The program involved: Reverse transcription reaction - 50°C for 10 minutes, denaturation at 95°C for 1 minute, 35 amplification cycles with denaturation for 10 sec and

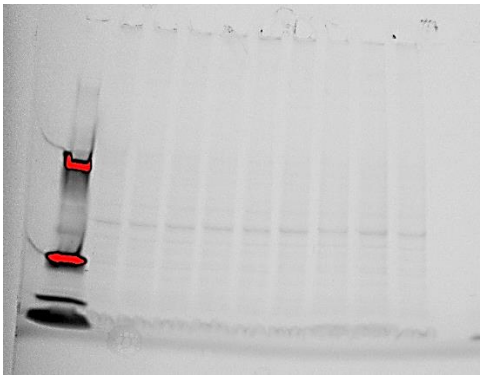
annealing/extension for 15 sec and finishes with 65°C for melt curve analysis (default setting was used).

Two qPCR was done to find the optimal primer pair with the highest PCR efficiency. 13 samples comprising the selected lines from monoclonal expansion was used as template. Primers used for first qPCR was Cas9, Cas9.1, GAPDH and HPRT. In second, Cas9, Cas9.3, GAPDH and TBP was used. Two pairs of standards were used (8 samples) having 1:4 dilutions each to create the calibration curve. The results were analyzed using both 2^–ΔΔCT (Livak) Method and Pfaffl method.

2.6. Western blotting

8 cell lines showing a range of Cas9 expression were selected for protein analysis. Stain free western blotting protocol was used and the samples were lysed in ice cold RIPA buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, Roche Protease inhibitor tablet). 20 uL of the lysate was used for protein assay. Qubit Protein BR assay kit (A50668) was used for total protein quantification. 20 ug of each sample was used with equal volume of 2X Laemmli buffer and boiled for 95°C.

Mini-PROTEAN TGX Stain-Free Precast Gels were used for protein separation and 10 ug of sample + buffer mixture was added in each well with running buffer (25mM Tris, 190mM glycine, 1%SDS, pH 8,3) and ran for 15 minutes at 200 V. The gel was then visualized a Chemidoc MP imaging System (Application: Stain-free gel, Gel Activation time: 45 sec, Image activation: Optimal automatic exposure) – **Image below**.



Bio- rad trans-blot Turbo transfer pack (1704156) was used for transfer. The gel was placed on top of the membrane sandwiched by the filter layers and kept inside the cassette. High MW transfer

was selected and ran for the designated time period. The blot and the gel were then kept in milli-Q water and used for imaging. The blot was imaged in Chemidoc MP imaging System (Application: Stain-free blot, activation: Optimal automatic exposure) and kept in TBST (20mM Triss, 150mM NaCl and 0,1 % Tween 20).

1% Casein was used as the blocking buffer and the blot was kept in it with constant agitation for 5 minutes. The blot was then cut into two parts based on the assumed molecular weights (MW) of Cas9 using Precision Plus Protein Kaleidoscope Standard. Primary antibody used in upper part (more MW) was Cas9 mouse monoclonal IgG (7A9-3A3) and was diluted in the ratio of 1:500 with the blocking buffer. Monoclonal anti-b-actin antibody (A228-100ul) was used as the primary antibody in the lower part and was diluted in the ratio of 1:10000. Both the blots were kept in 4°C with constant agitation overnight.

The blots were then washed with TBST, 5 times each for 5 minutes. Horseradish peroxidase (HRP)-conjugated secondary antibody was diluted in 1:10000 ratio with blocking buffer. The blots were rinsed together in it for 1 hour at room temperature and then washed six times with TBST for 5 minutes and incubated in Clarity Max substrates (1:1 ratio) for 5 min. The blots were then imaged separately in in Chemidoc MP imaging System (Application: Chemiluminescent Blot, activation: Optimal automatic exposure). Protein normalization was done using volumetric tools. Band intensities were calculated for both the blots after adjusting the background and then normalized Cas9 band volumes with actin band volumes to get the normalized values.

2.7. Target guide sequence design

Guides targeting ADAM10 as well as non-targeting controls were picked from the house designed library.

sgRNA	**Oligo 1**	**Oligo 2**
ADAM 10_2 (AD2)	*CACCGATCTCGATCTGTGAAAACGT*	*AAACACGTTTTCACAGATCGAGATC*
ADAM10_3 (AD3)	*CACCGCCCATAAATATGGTCCACAG*	*AAACCTGTGGACCATATTTATGGGC*
BT104nt1 (NT1)	*CACCGCGAATGATCCGGGTCTACTG*	*AAACCAGTAGACCCGGATCATTCGC*
BT104nt2 (NT2)	*CACCGCCATACCATCGAGGGCTTAG*	*AAACCTAAGCCCTCGATGGTATGGC*

Table 3: sgRNA sequence oligos with overhangs

2.8. Creating lentiviral sgRNA plasmid

Optimised production of lentiviral sgRNA plasmids is essential as it directs the Cas9 cleavage. LentiGuide-Puro (Plasmid #52963) was used to create CRISPR plasmid. Optimized 'LentiCRISPRv2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA' protocol (Shalem et al., 2014) was used. The concentration of LentiGuide-Puro was determined using a NanoDrop and 50 ng was used for each guide RNAs. Four guides as represented in the above Table 3 was used as a pair of annealed oligos, to be cloned to the single guide RNA scaffold in the LentiGuide-Puro plasmid.

2.8.1. LentiGuide-Puro Digestion and dephosphorylation

5 ug of plasmid was digested using 3 uL of BsmBI (R0580L) and dephosphorylated using FastAP in 6 uL of 10X rCutSmart Buffer. The solution is made upto 60 uL using milli-Q water and used to run in an agarose gel for separation of plasmid backbone and cut out site. QIAGEN gel purification kit was used to obtain plasmid backbone.

2.8.2. Phosphorylation and annealing of guides

Each pair of guide oligos (1 uL of 100 uM) were annealed and phosphorylated using 1 uL of 10x T4 Ligation buffer (B0202A) and 0.5 uL of T4 PNK (M0201S). The total reaction volume was made up to 10 ul. The mixture was kept in a thermocycler and ran using the following parameter causing the annealing of oligos: 37°C for 30 min; 95°C for 5 min and ramping down to 25°C at 5°C/min. The annealed guide oligos were then diluted to 1:200 dilution using milli-Q water.

2.8.3. LentiGuide-Puro ligation and transformation

50 ng of digested LentiGuide-Puro plasmid and 1 ul of diluted oligo duplex is mixed along with Quick Ligase. Negative control ligation was also done using vector + water. All the duplex guide oligos were then transformed to Stbl3 bacteria. Transformation was done using heat shock, 20 uL of Stbl3 bacteria was added along with 1 uL of of ligation product and kept in ice for 30 min. Then the cells were heat shocked in a water bath of 42°C for 45 seconds. The vials were then kept back on ice and 180 uL of LB (Luria-Bertani media) was added and kept in 37°C for 1 hour. After an hour each vial with different ligation products were spread in a LB plate containing ampicillin and kept overnight in incubation (37°C).

2.9. Colony PCR screening and Plasmid isolation

For identifying positive colonies from the plate, a colony PCR was done. Random colonies were streaked using a pipette and inoculated in the master mix as template for amplification. Platinum™ II Hot-Start Green PCR Master Mix (2X) was used. The PCR program was pretty much the same except the initial denaturation step (94°C – 1 min, 98°C – 5 sec, 55°C – 5 sec, 72°C-40 sec). The PCR products were run in a gel to identify the positive clones. Positive clones were then picked from the plates and inoculated in LB media and kept in a shaking incubator (37°C) overnight. 11 positive colonies were separately grown and used as samples for plasmid DNA isolation. QIAprep Spin Miniprep Kit (1000) (27106X4) was used for DNA isolation and then checked for purity and concentration. The sgRNA inserts were then confirmed through sanger sequencing.

2.10. Transduction and selection for puromycin resistant cells

LentiGuide Puro plasmids obtained from sgRNA inserts ('Lentivirus packaging' section) were then used to transduce selected 5 Cas9 MDBK cell lines. The transduction method/selection is same as recorded in the above section except the antibiotic used. Puromycin was used for selection. Each cell line had 5 transductions (CMV-GFP, AD2, AD3, NT1, NT2) and 3 from these were selected (AD2, AD3, NT1) from each line and used for PCR. AD2 and AD3 locus were amplified in all these three lines. A PCR purification was done for the detected bands in the gel and then send for sequencing. Primers used for amplification and sequencing as in the same order in **Figure 12** is provided in **Table 4.**

Samples	**Loci amplified**	**Primer (F)**	**Primer (R)**	**Sequence primer**
AD2	ADAM10_2	*ACTGCTGATGAGAAGGACCC*	*TAAGAACCTCTGCCCAACCC*	*CGGGCATCACTTTCTCCTATAC*
AD2	ADAM10_3	*GCATTGGGAGCATGGTTTCT*	*GGGGTCATGCAGATTGTATTCA*	*GCAGAAACACACGCACAACT*
AD3	ADAM10_2	*ACTGCTGATGAGAAGGACCC*	*TAAGAACCTCTGCCCAACCC*	*CGGGCATCACTTTCTCCTATAC*
AD3	ADAM10_3	*GCATTGGGAGCATGGTTTCT*	*GGGGTCATGCAGATTGTATTCA*	*GCAGAAACACACGCACAACT*
NT1	ADAM10_2	*ACTGCTGATGAGAAGGACCC*	*TAAGAACCTCTGCCCAACCC*	*CGGGCATCACTTTCTCCTATAC*
NT1	ADAM10_3	*GCATTGGGAGCATGGTTTCT*	*GGGGTCATGCAGATTGTATTCA*	*GCAGAAACACACGCACAACT*

Table 4: samples and their primers for PCR amplification and sequencing

2.11. Sequence alignment and editing efficiency

All the samples were sent to Europhins genomics (https://eurofinsgenomics.eu/) for sanger sequencing as an internal generated order. All the samples were premixed with 5nM internal sequencing primer in barcoded tubes from the Mix2Seq kit. The sequence data is sent back as a zip file with PDF, FASTA and ABI format which is used for further analysis.

Sequence alignment was checked using online platform Benchling (https://benchling.com/). For confirming the sgRNA integration, all the target sequences were aligned to lentiGuide puro sequence using internal sequence alignment function. Same function was also used to check the aligned sanger sequencing traces of the edited loci after performing CRISPR/Cas9 editing. Editing efficiency was determined using Tide (https://tide.nki.nl/) which employs decomposition based algorithm to assess genome edits (Brinkman et al., 2014). Edited lines along with control and the gRNA sequence (excluding PAM) was uploaded to the pipeline to generate editing efficiency with indel data.

3. Results

The entire research methodology (**Figure 3**) described in the above section spanned about 6 months until any valid results were received. The research method involved a series of steps which were either done simultaneously or as in a workflow.

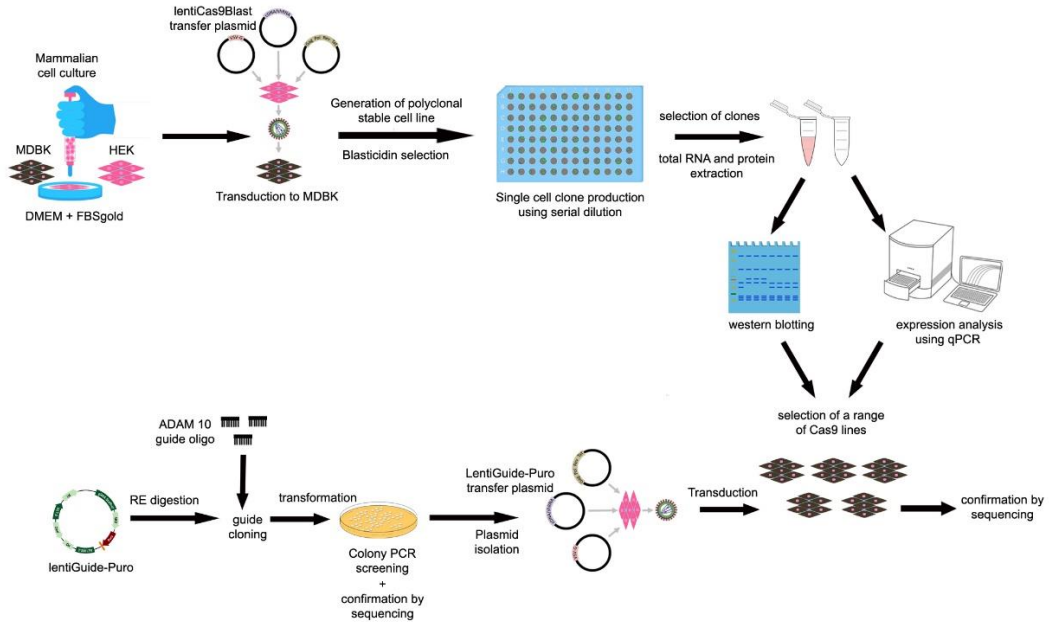


Figure 3: Diagrammatic representation of the entire methods used in this study

3.1.Production of polyclonal and monoclonal cell lines

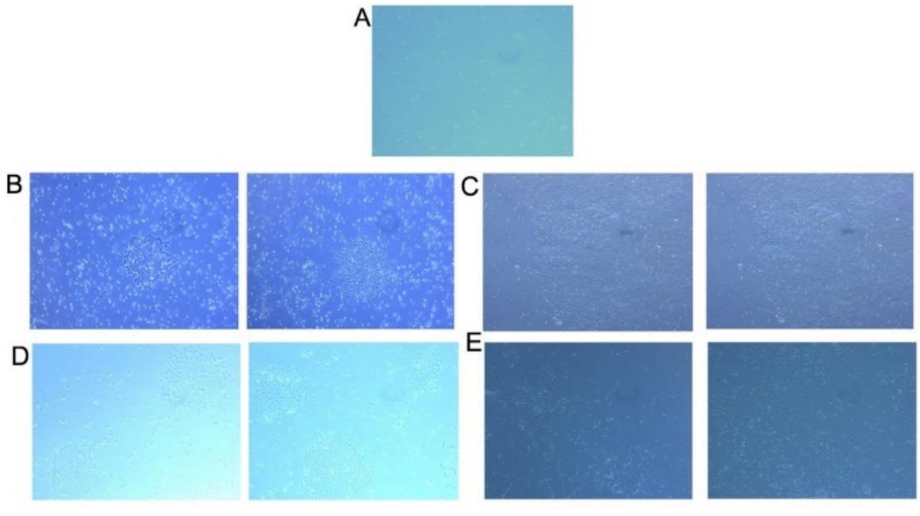


Figure 4: A: Mock infected; **B** panel: Batch 1 MDBK cells transduced using virus with pMD2.G – after 5 days; **C** panel: Batch 1 MDBK cells transduced using virus with pMD2.G – after 9 days; **D** panel: Batch 2 MDBK cells transduced using virus with pMD2.G – after 5 days; **E** panel: Batch 2 MDBK cells transduced using virus with pVSV-G – after 5 days

LentiCas9 blast cells obtained following blasticidin selection produced stable cell lines as compared to the no virus control (Mock infected) (**Figure 4** A-C). Using two different envelope plasmids (pMD2.G and pVSV-G) didn't conferred a significant difference in cell growth rate (**Figure 4** D, E). Serial dilution produced 21 single cell clones (**Figure 5**) in a total of four 96-well plates and was expanded to T75 flasks. After 2 passages, 13 lines (named as L1-L13) were selected randomly along with a negative control line (NC) for further downstream processes.

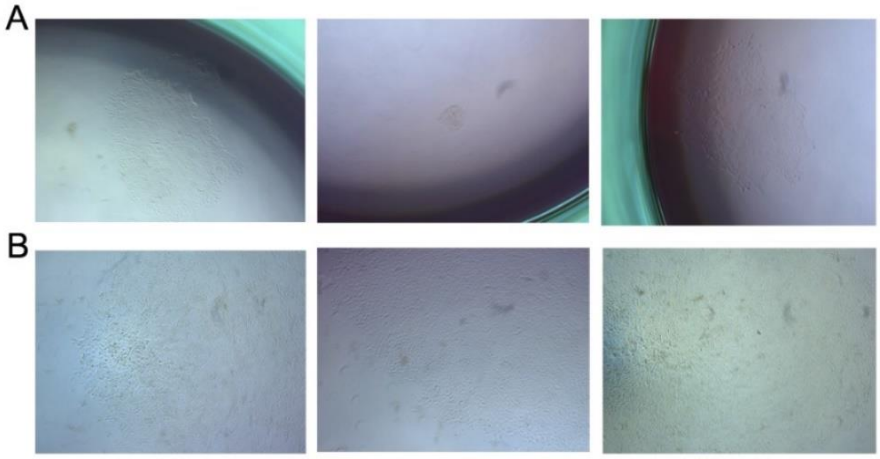
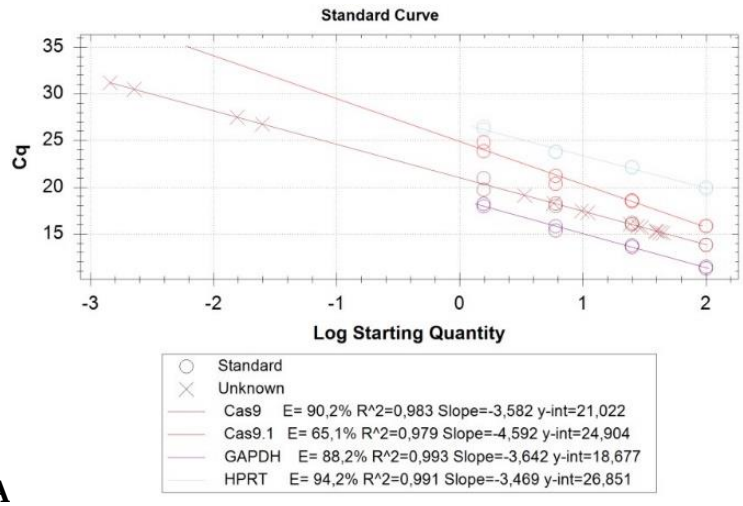


Figure 5: A panel: single cell colonies after 9 days; **B** panel: single cell colonies after 11 days

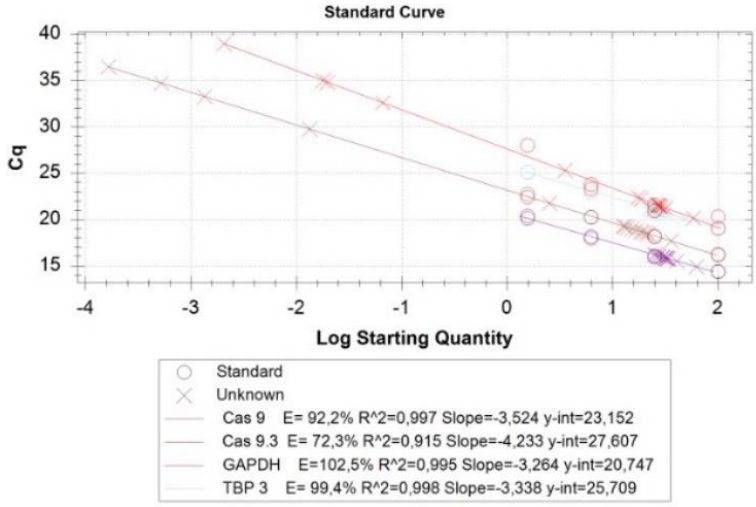
3.2. qPCR analysis

Two different qPCR analysis was done using 6 primers (3 target genes: Cas9, Cas9.1, Cas 9.3; 3 reference genes HPRT, GAPDH, TBP) to determine the optimal primer pair. Amplification efficiency (E) could be calculated from the slope of calibration curves (**Figure 6**) using the formula E = 10^(-1/slope). And percentage efficiency could be calculated by: % Efficiency = (E-1) x 100

In the first qPCR experiment, out of the 4 probes used, the primer pairs selected for quantification was Cas9-HPRT. Cas9.1 had very less %E and was not used for quantification. GAPDH even though had similar efficiency with Cas9 but was not selected since it wasn't close to 100%. Cas9-HPRT was selected as the amplification efficiency of both these genes are similar (difference less than 5%) and close to 100%. Here in this case, Livak method was used for calculating relative gene expression.



A



B

Figure 6: Calibration curves from the CFX Maestro software: **A**: First qPCR experiment; **B**; Second qPCR experiment

In the second qPCR done for validating the first experiment, the target gene selected was Cas9 and the reference genes GAPDH and TBP. Amplification efficiency of Cas9.3 not close to 100% and thus was not used for quantification. Even though Cas9 had closer efficiency towards the reference probes as compared to Cas9.3, since it was not having similar efficiency (difference of 5%), Pfaffl method was used for relative quantification.

13 lines obtained from monoclonal culture expansion were used as the test samples. The Lines were named in the same order it was harvested temporally from the plates for RNA and protein extraction and then kept constant. **Figure 7** shows the relative expression of Cas9 with respect to HPRT. Line 4 (L4) was selected as the calibrator and all other lines are compared relative to it.

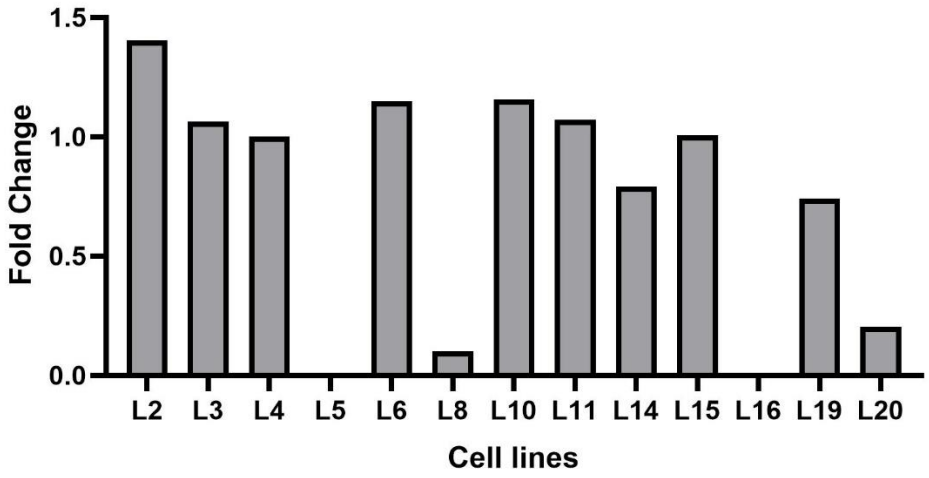


Figure 7: Relative expression of Cas9 in different cell lines compared to HPRT reference gene.

The second qPCR experiment was done with reference to GAPDH and TBP (**Figure 8**). The data aligned with the absence of expression in two of the lines (L5 and L16). The detectable line set for callibration was also L4. The expression showed similar trends but the fold change was not comparable probably due to the reference gene difference. There was no sample in the well with L20, so it was discarded.

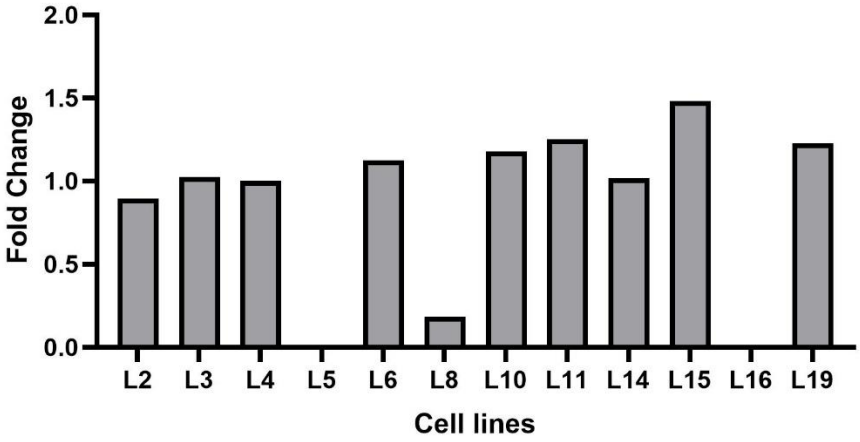


Figure 8: Relative expression of Cas9 in different cell lines compared to GAPDH and TBP reference genes.

8 lines were selected based on the qPCR results which showed a range of expression: L2, L3, L4, L6, L8, L10, L15, L20 and used for confirming the Cas9 protein expression using Western blotting.

3.3.Western blotting

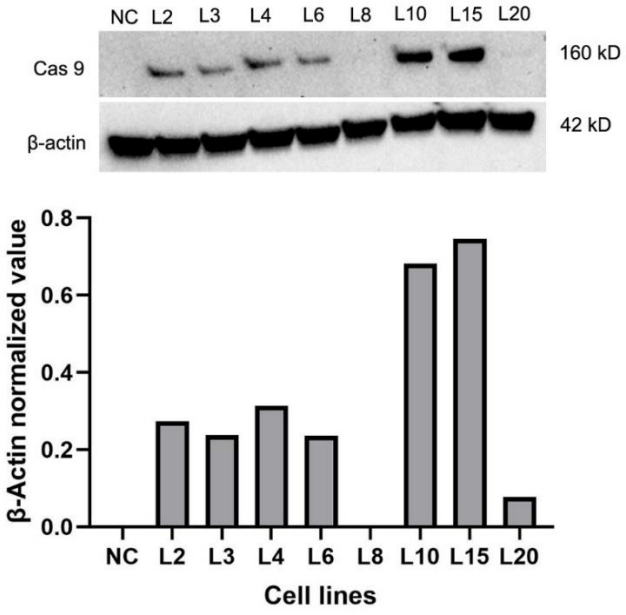


Figure 9: Upper pannel: Chemiluminescent western blot of both Cas9 and β-Actin; Lower Panel: Cas9 protein values normalised with β-Actin

Western blotting provided comparable results as with the first qPCR experiment. L8 and L20 were having little or no bands which aligns with the QPCR data. L10 and L15 is showing high protein expression which is comparable to the first qPCR result (**Figure 9**) but that is not the case for L2. The samples were loaded with the same concentration, therefore the changes in band intensities would be proportional to the protein concentration.

5 of the lines which showed a comparable range of Cas9 RNA and protein expression were selected for transduction with lentiviral vectors comprising sgRNA.

3.4.Colony PCR screening and confirmation of presence of sgRNA

Positive clones from the transformed Stbl3 bacteria selected using colony PCR. 8 colonies from each plate were screened for positive clones (**Figure 10**). The one with bands showed the presence of specific guide. The presence of sgRNAs was further confirmed by sequencing. The received sequences were aligned using Benchling to confirm the presence of sgRNA (**Figure 11**).

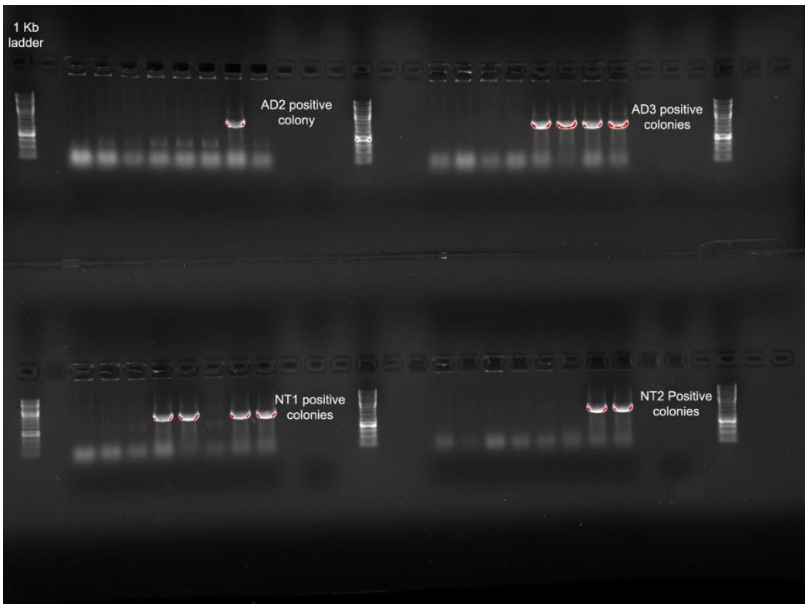


Figure 10: sgRNA positive colonies obtained from colony PCR

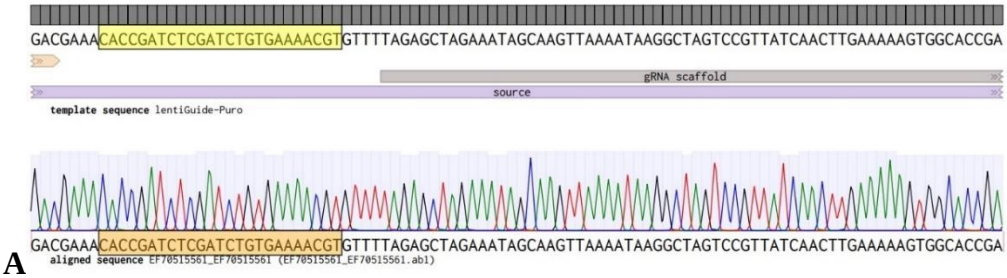


Figure 11: Sequences aligned with lentiGuide-Puro as template (upper); Both sgRNAs are highlighted along with the CACCG overhangs **A**: AD2 sgRNA; **B**; AD3 sgRNA

3.5. Editing confirmation and efficiency

The presence or absence of editing is confirmed using sanger sequencing. For that a PCR was done in 5 lines transduced with sgRNA lentivirus. The locus containing the edit were amplified and 3 lines (L4, L8, L10) showed positive results (**Figure 12**).

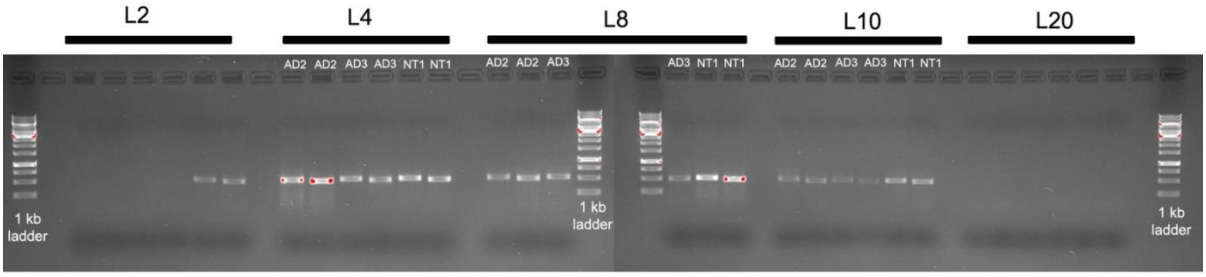


Figure 12: PCR performed in selected lines using primers described in **Table 4**. Bands are seen in all the samples for 3 lines. In LINE 2, only bands are seen in NT 1.

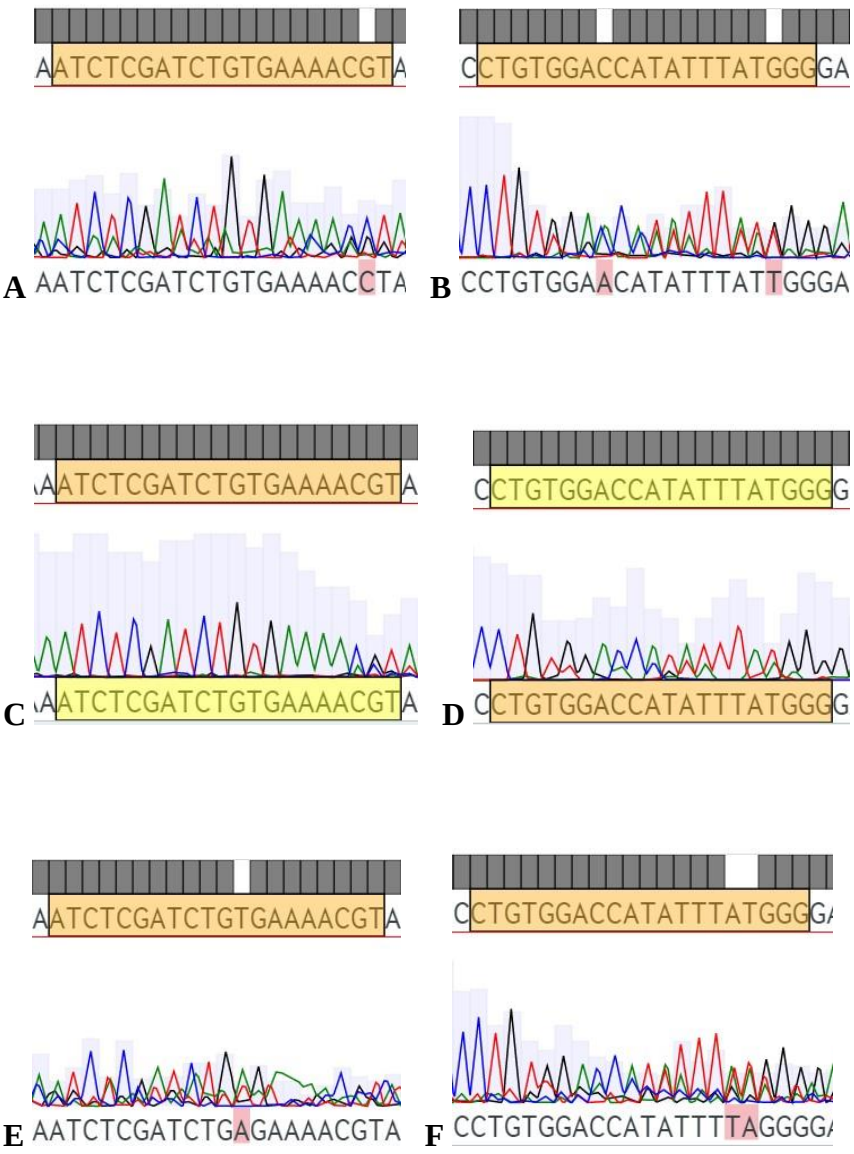


Figure 13: Each sgRNA loci highlighted with yellow/lime yellow color. Upper sequences are the non-targeted control. Traces of edit/mismatch denoted by red highlight over bases. **A**: Line 4/AD2 edited; **B**: Line 4/AD3 edited; **C**: Line 8/AD2 edited; **D**: Line 8/AD3 edited; **E**: Line 10/AD2 edited **F**: Line 10/AD3 edited

Each cell line comprised specific AD2 edited and unedited cell lines; AD3 edited and unedited cell lines. L4, L8 and L10 were then sequenced and checked for traces of edits compared to the unedited loci.

In Line L4 and L10 there are traces of edits at least in one or two base pairs. L8 on the other hand shows no traces of edits (**Figure 13**). L4 and L10 were further analyzed for sequence efficiency and indel proportions using Tide (**Figure 14**).

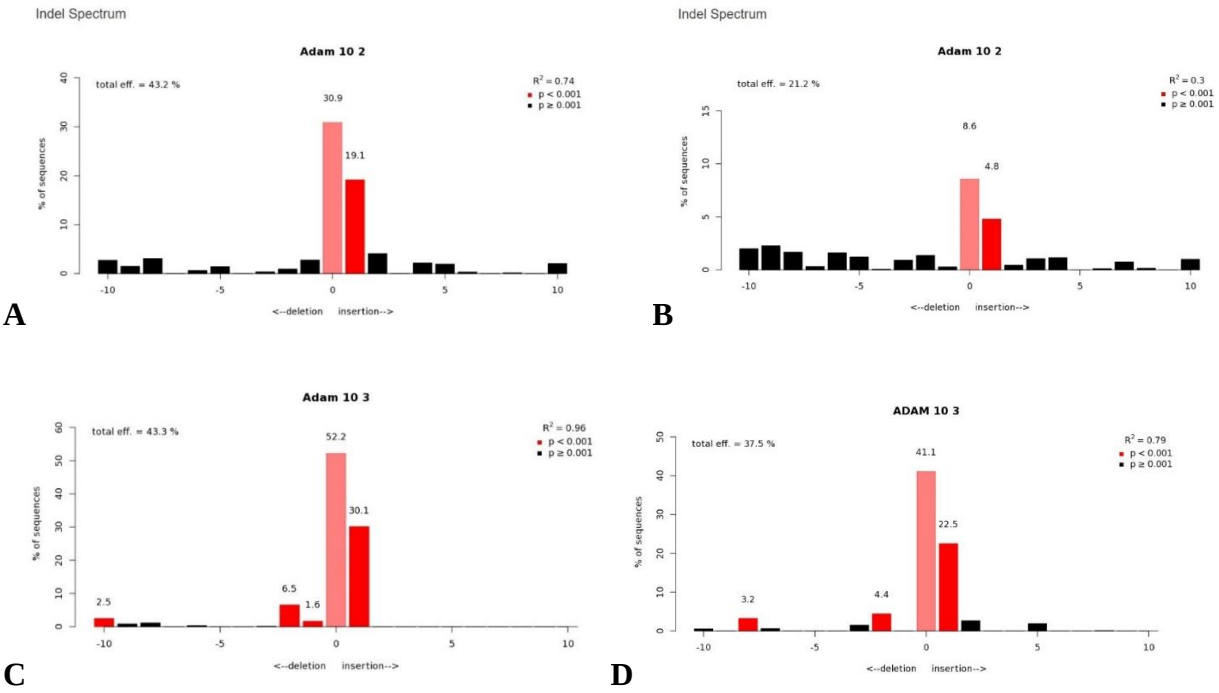


Figure 14: spectrum of indels in each edited cell lines **A**: Line 4/AD2 edited; **B** Line 10/AD2 edited; **C**: Line 4/AD3 edited; **D**: Line 10/AD3 edited

Discussion

The rationale of this work mainly involved developing Cas9 integrated cell lines for Genome-wide CRISPR-Cas9 knockout screens and to correlate the editing efficiency of selected lines to its Cas9 expression. The primary aim was always the successful development of Cas9 expressing robust lines. Great interest was provided into the expression profiles and protein quantification to confirm that. These lines will then be used for the subsequent lentiGuide-Puro (two vector system) GeCKO screening. Any information regarding the Cas9 expression and editing efficiency was the secondary objective if enough edited samples were obtained.

Cas9 expression of different lines had comparable correlations with the protein data. Increased protein levels were observed in LINE 10 and the qPCR data reinforces it. Similarly, the no-blot scenario in Line 8 and less protein quantity in LINE 20 was correlated. Our lines of interest, L4 and L10 had similar RNA levels but different protein quantities. The Cas9 protein level was higher in L10 as compared to L4.

One reason could be the activity of any post-transcriptional regulation mechanisms specific for a cell line. Quality of the antibody used in the assay may also be a factor that could affect the correlation. High sensitivity of qPCR may also be a factor for overexpression of Cas9 in certain lines.

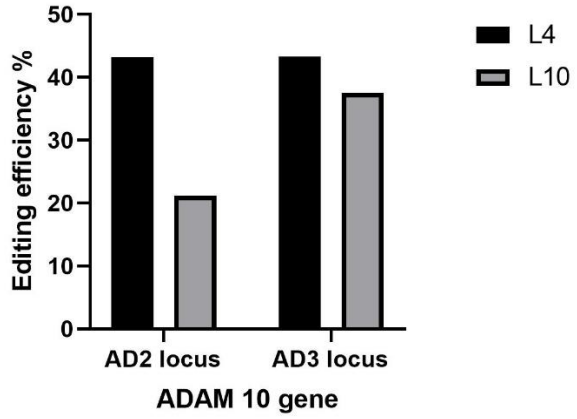


Figure 15: Editing efficiencies compared between 2 lines

CRISPR/Cas9 editing

Transduction of the selected lines with lentivirus containing sgRNA results in the integration of guides into the cells. This results in the targeted gRNA-based editing using the existing Cas9

machinery. The scope of this thesis is also to analyze this editing efficiency with the quantified Cas9 levels.

From the selected 5 lines for a range of protein levels, experimental validations were limited to 3 because of failed locus amplification. And out of those lines, only two were having base edit/mismatch while aligning. Comparing the editing efficiencies, LINE 4 had higher efficiencies for both the loci (43%) as compared to Line 10 (**Figure 15**). It is also observed that Line 4 had more deletions compared to Line 10.

Since LINE 4 had lower protein and comparable RNA levels with LINE 10, we could not point out that higher Cas9 levels can always contribute towards higher editing efficiencies. Instead, we could postulate that an optimal level of Cas9 expression could result in better editing efficiency. However, it is difficult to infer any validations from just two lines.

Conclusion

The primary objective of the study was fulfilled by the generation of Cas9 expressing MDBK cell lines for subsequent screening process. The lines will now be used for GeCKO screening against BVDV and BCoV.

Based on the samples obtained, the editing efficiency was not directly depended upon the Cas9 expression or protein levels. Given that, more sample size and correlation statistics are needed to validate the finding.

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