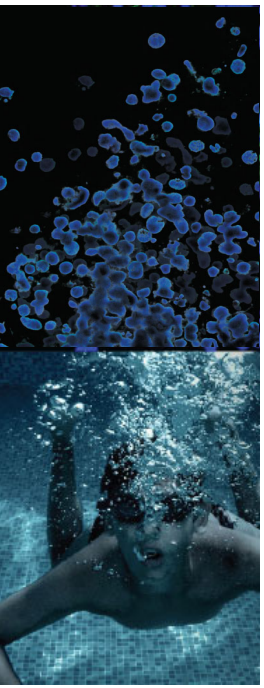
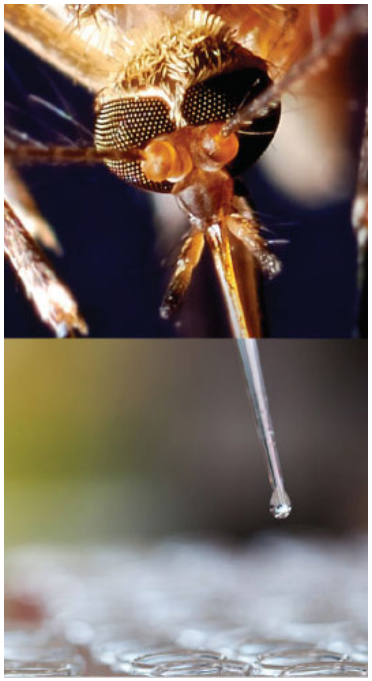
Amplify Your Viral Vaccine Production with CRISPR/Cas9 Engineered Host Cells

Liz Turner Gillies, PhD





Credible Leads to Incredible™

About ATCC

- Founded in 1925, ATCC is a non-profit organization with HQ in Manassas, VA, and an R&D and Services center in Gaithersburg, MD
- World's largest, most diverse biological materials and information resource for microbes – the "*gold standard*"
- Innovative R&D company featuring gene editing, microbiome, NGS, advanced models
- cGMP biorepository
- Partner with government, industry, and academia
- Leading global supplier of authenticated cell lines, viral and microbial standards
- Sales and distribution in 150 countries, 19 international distributors
- Talented team of 450+ employees, over one-third with advanced degrees

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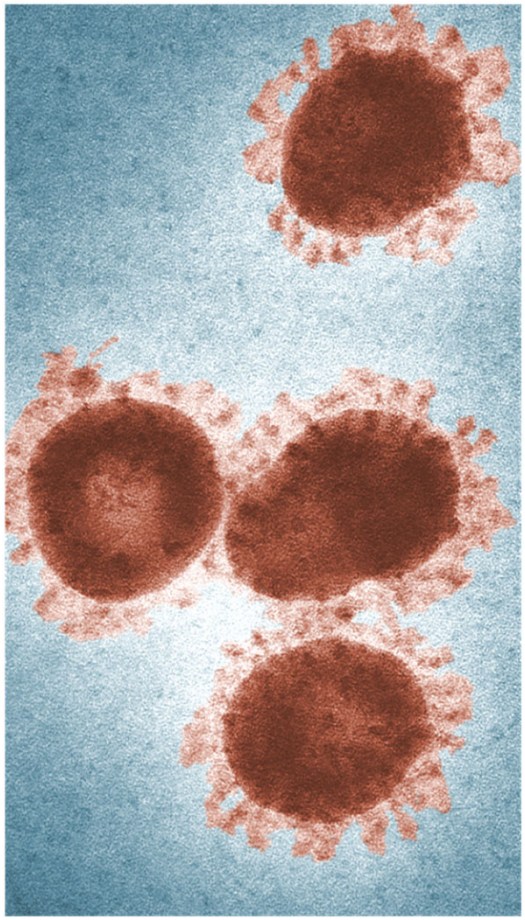
Outline: CRISPR-Cas9 Engineered Viral Host Cells



I. Introduction to viral vaccine production and CRISPR/Cas9 gene editing

II. Gene editing of Vero cells for enhanced viral production

III. Gene editing of MDCK cells for enhanced viral production

Coronavirus, image courtesy of CDC Public Health Image Library

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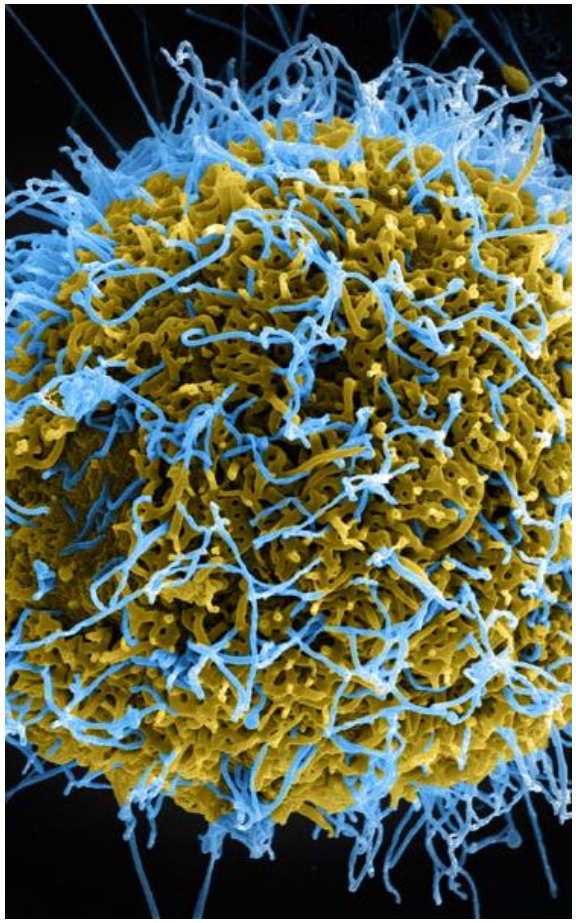
I. Introduction to Vaccine Production and Gene Editing

- Overview of viral vaccines
- Manufacturing of viral vaccines in host cell culture
- Cell lines used for viral vaccine manufacturing
- Opportunity for improvement of viral production cells
- Technology for cell line modification
- Host cell anti-viral response
- Use of NHEJ repair mechanism for gene knockout
- Development of enhanced viral production cell lines



Ebola virus infecting Vero cell, image courtesy of NIAID

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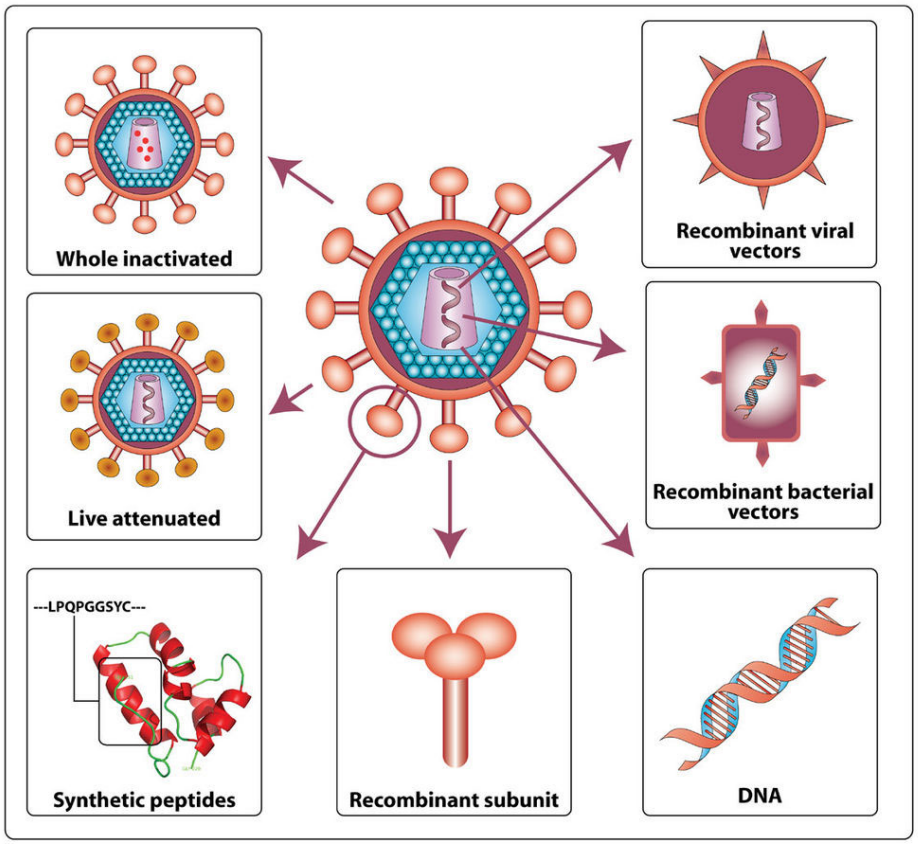
Overview of Viral Vaccines

Vaccines against viral infections are designed to introduce your immune system to that virus without actually making you sick



en.wikipedia.org/wiki/HIV_vaccine

Examples of Viral Vaccines

- Flu shot (Influenza)
- MMR (Measles, mumps, rubella)
- Varicella (Chickenpox)
- Polio vaccine
- HPV (Human papillomavirus)
- HepA/B/C (Viral hepatitis)
- Rotavirus vaccine
- Rabies vaccine

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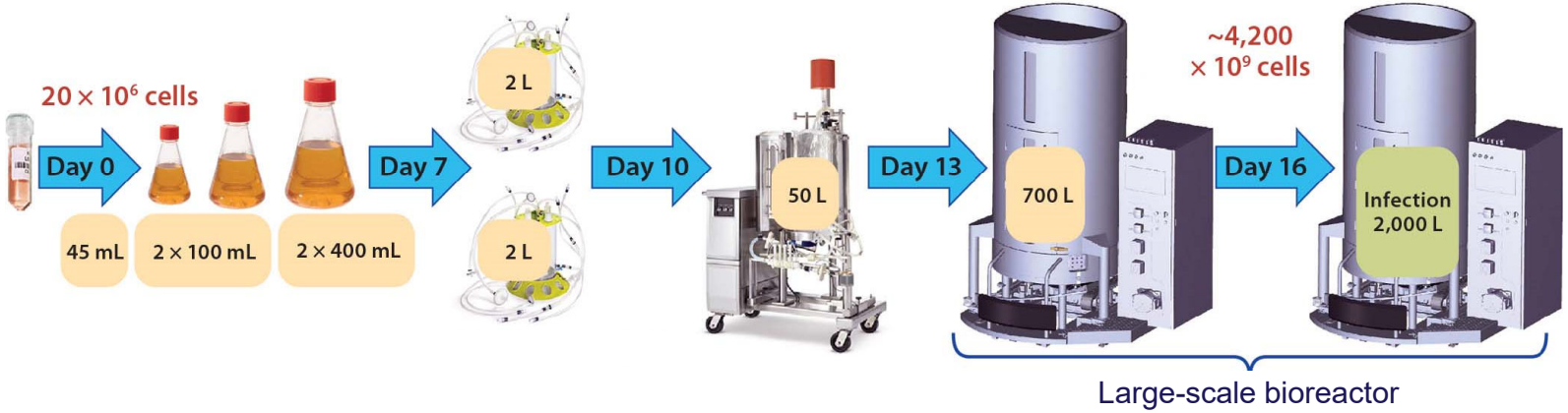
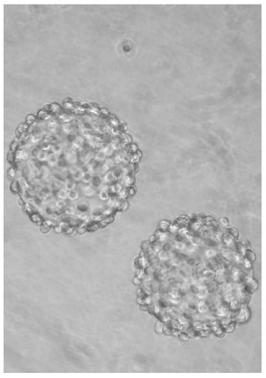
Manufacturing of Viral Vaccines in Host Cell Culture

Viral products for vaccines are primarily manufactured in large-scale tissue culture systems

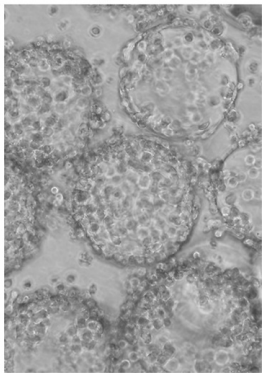


Adherent cells on micro-carrier beads

Vero cells

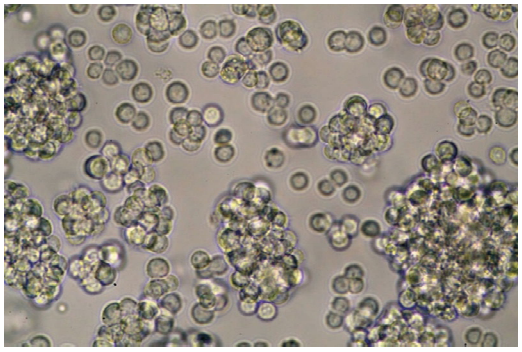


MRC-5 cells



PLoS ONE 3(3):e1810

Carrier-free suspension culture



bioprocessintl.com/upstream-processing/upstream-single-use-technologies

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Cell Lines Used for Viral Vaccine Manufacturing

A handful of traditional cell lines are used for viral vaccine manufacturing

Name	ATCC® No.	Species	Cell type	Year Isolated	Vaccines
MDCK	CCL-34™	*Canis familiaris* (domestic dog)	Kidney epithelial	1958	Influenza A, influenza B (seasonal)
Vero	CCL-81™	*Cercopithecus aethiops* (green monkey)	Kidney epithelial	1962	Rotavirus, vaccinia, polio, rabies, Japanese encephalitis, dengue, Zika, Chikungunya
WI-38	CCL-75™	Homo sapiens (human)	Lung fibroblast	1961	Adenoviruses, rubella, measles, mumps, varicella zoster, polio, Hep A, rabies
MRC-5	CCL-171™	*Homo sapiens* (human)	Lung fibroblast	1966	Zoster, polio, hepatitis A, hepatitis B, Varicella, measles, mumps, rubella, rabies

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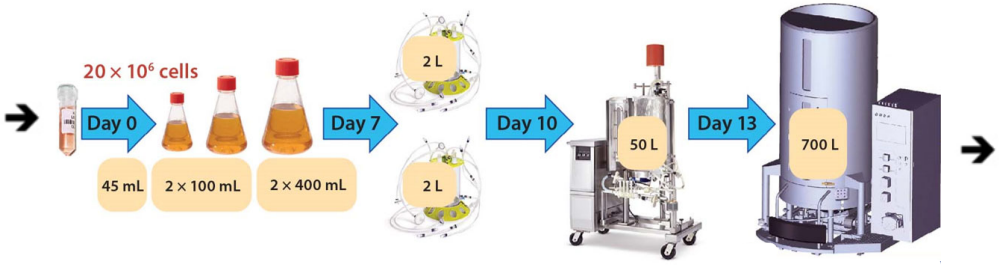
Opportunity for Improvement of Viral Production Cells

Can cell lines be enhanced to produce viruses more efficiently?

Current vaccine production cell line



Improved vaccine production cell line

- Faster virus production
- Smaller production scale required to meet manufacturing goals
- Lower production costs
- Fewer regulatory hurdles for using new version of approved cell line than for a new cell line

bioprocessintl.com/upstream-processing/upstream-single-use-technologies

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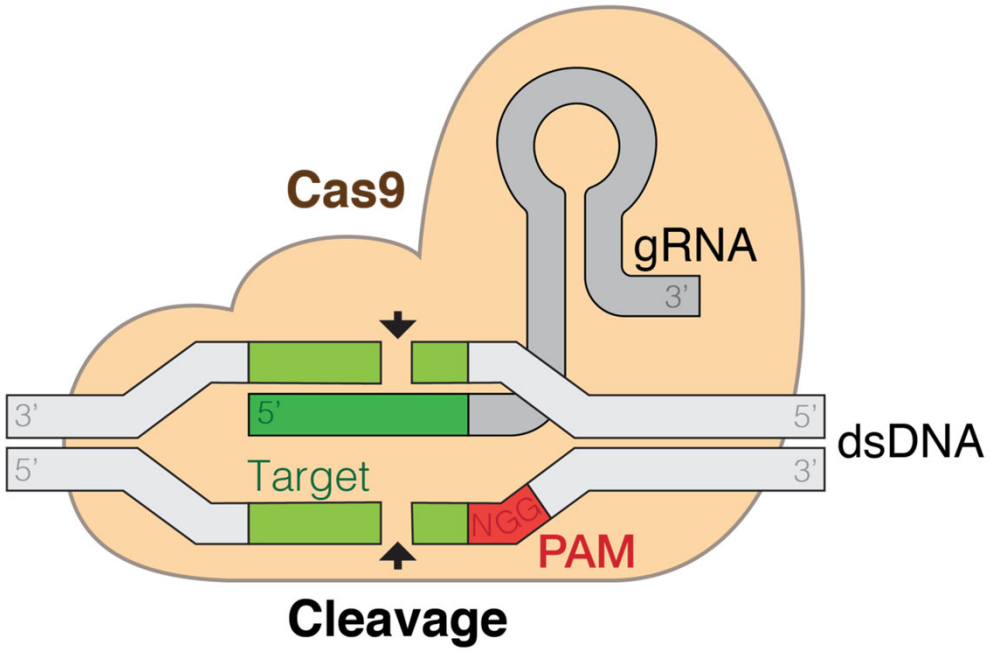
Technology for Cell Line Modification

CRISPR-Cas9 can be used to permanently change the genetics and characteristics of a cell line



CRISPR-Cas9 can be used to:

- Locate specific gene sequences inside the nucleus of a cell
- Cut the genomic DNA at that specific location
- Change the genetic sequence at the cut site (mutation)
- Add new genetic sequence to the cut site (insertion)
- Remove genetic sequence from the cut site (deletion)
- Permanently change the characteristics of the cell controlled by the modified gene

en.wikipedia.org/wiki/CRISPR_gene_editing

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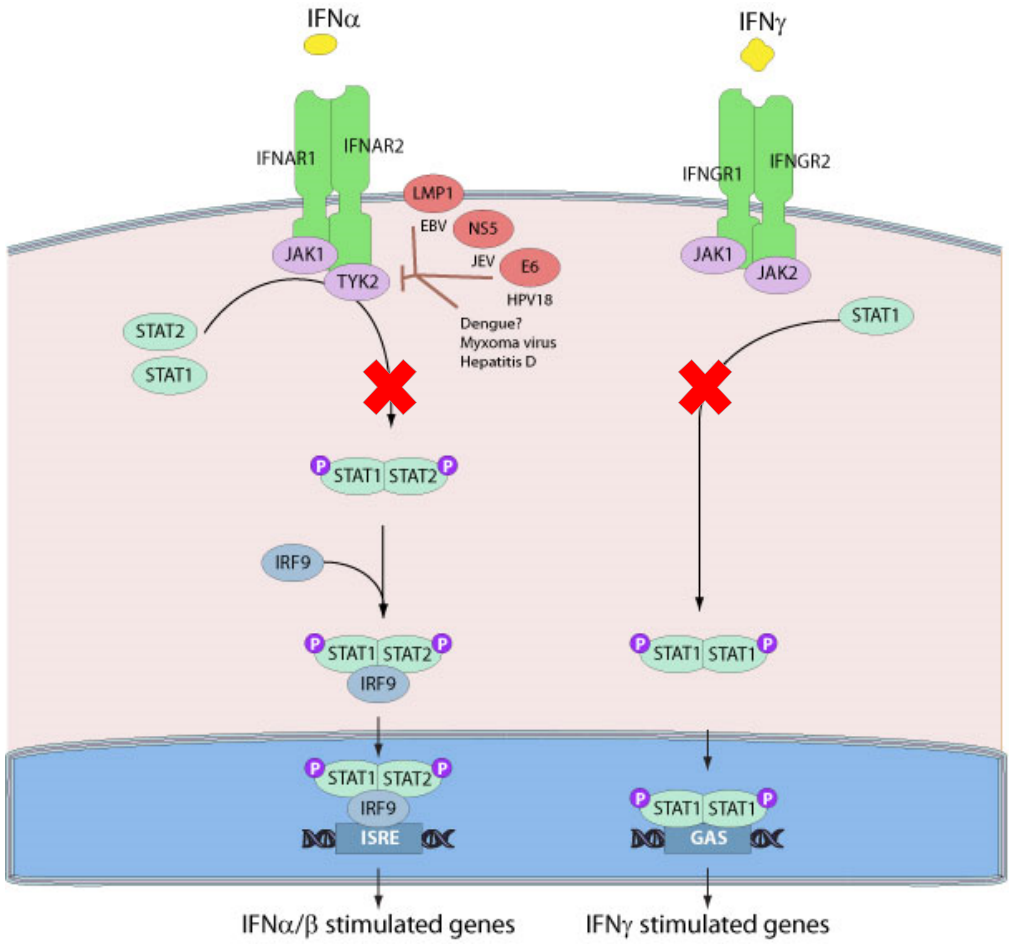
STAT1 Signaling Controls Host Cell Anti-viral Response



- Cell lines used in viral production have anti-viral interferon response
- STAT1 protein is essential for anti-viral interferon response
- Strategy – use CRISPR/Cas9 to disrupt STAT1 gene in cells used for viral production
- No STAT1 protein is produced when STAT1 gene is disrupted
- Virus production is enhanced in STAT1 deficient cell lines

viralzone.expasy.org – inhibition of host STAT1 by virus

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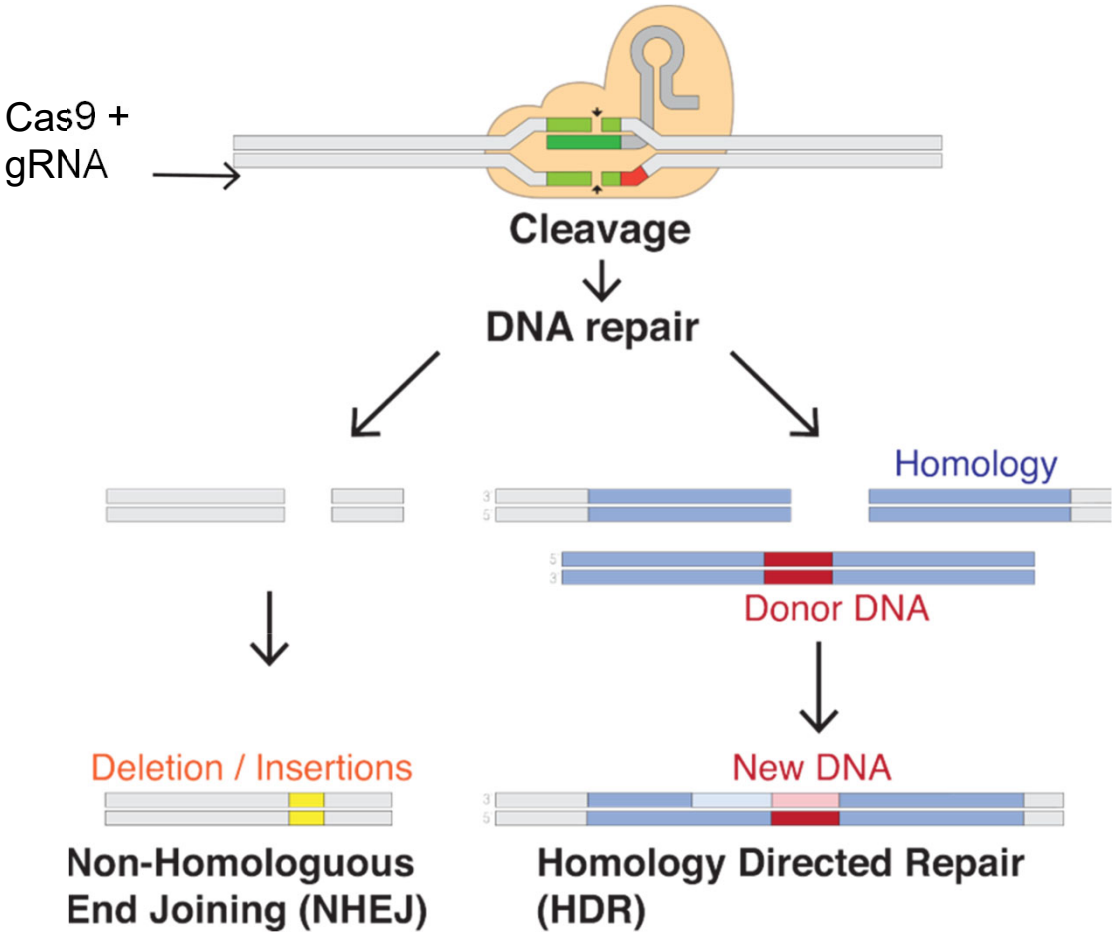
Use of NHEJ Repair Mechanism for Gene Knockout



- Design and construct CRISPR/Cas9 reagents to target STAT1 gene
- Transient transfection of Cas9 and STAT1 gRNA constructs into viral production cells
- CRISPR/Cas9 creates double-strand break (DSB) in STAT1 gene using
- DSB is repaired by non-homologous end joining, an error-prone cellular DNA repair mechanism
- NHEJ results in small insertions and deletions at repair site
- Out-of-frame insertions and deletions near the start of STAT1 gene result in a functional protein knockout

en.wikipedia.org/wiki/CRISPR_gene_editing

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Development of Enhanced Viral Production Cell Lines

Evaluate host cell lines

Select target genes

Edited cell pool test
- Editing efficiency
- Functional test

Single clone screening
- Sequencing
- Bio-functional screening

Select top clones
- Sequence verification
- Transcript testing
- Protein expression
- Off target edit evaluation
- Stability testing
- Morphology and cell growth
- Bio-function evaluation

Enhanced vaccine production cell models

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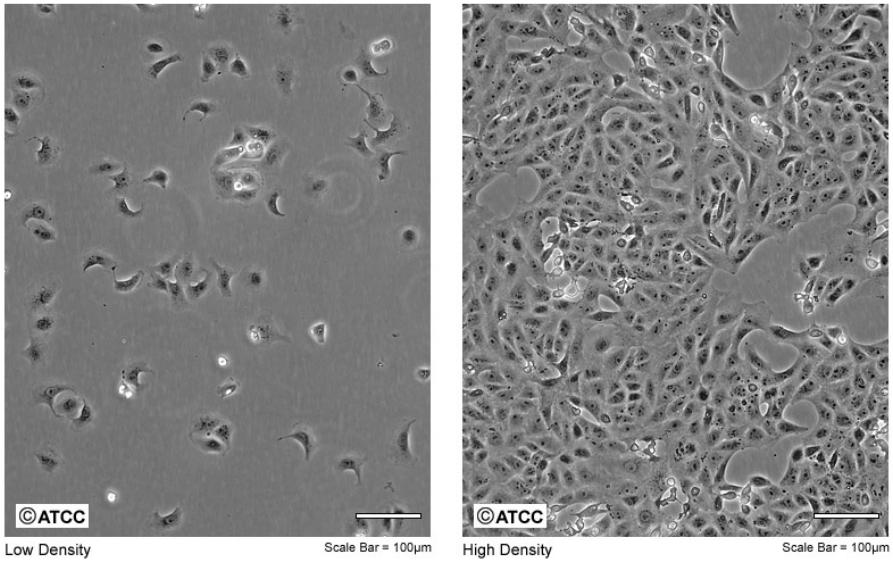
Viral Vaccine Production in Vero Cells

ATCC Number: **CCL-81**
Designation: **Vero**



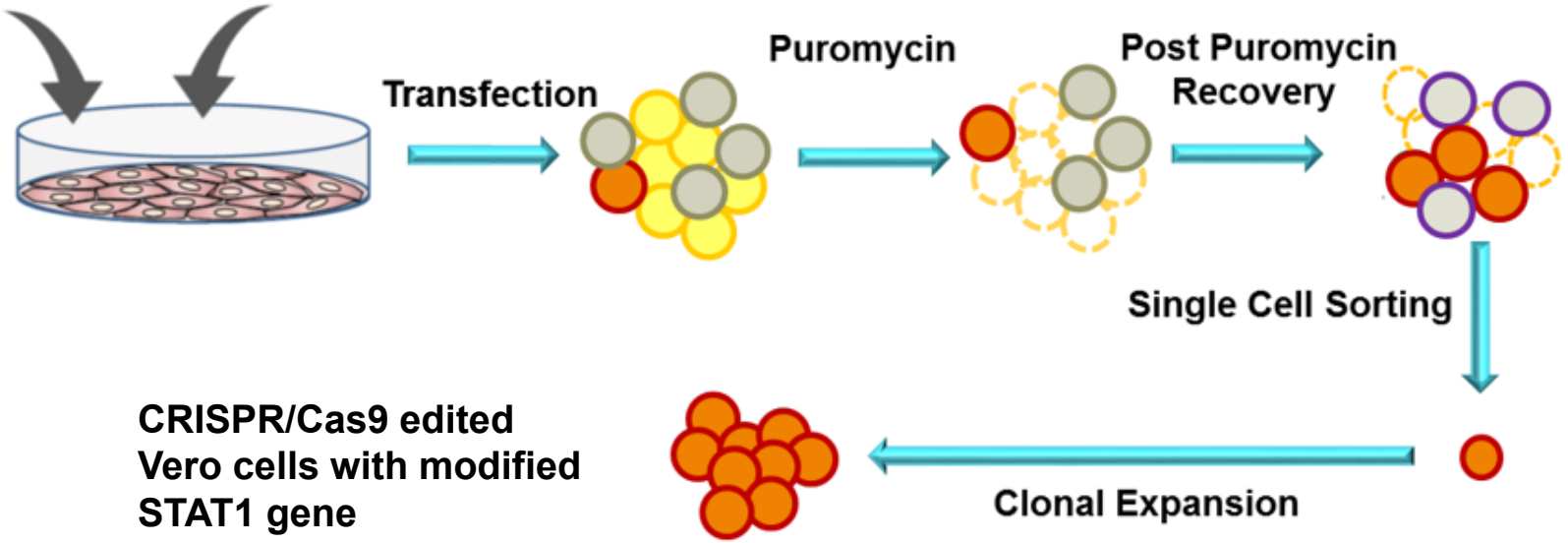
- Vero is an adherent epithelial cell line established from the kidney of a normal adult African green monkey in 1962.
- Vero cells are unusually permissive to infection by a wide variety of animal viruses.

- Vero remains one of the most commonly used cell lines for cell-based viral vaccine production.
- Vero cell line has received worldwide regulatory acceptance.
- Abundant studies on the production of a variety of viruses using Vero cells, such as:
  - Rotavirus
  - Influenza
  - Vaccinia
  - Polio
  - Rabies virus
  - Japanese encephalitis virus
  - Dengue
  - Zika
  - Chikungunya.

Workflow of CRISPR/Cas9 Gene Editing

Used to modify the STAT1 gene of Vero cells

Cas9-Puro and guide RNA targeting STAT1 gene

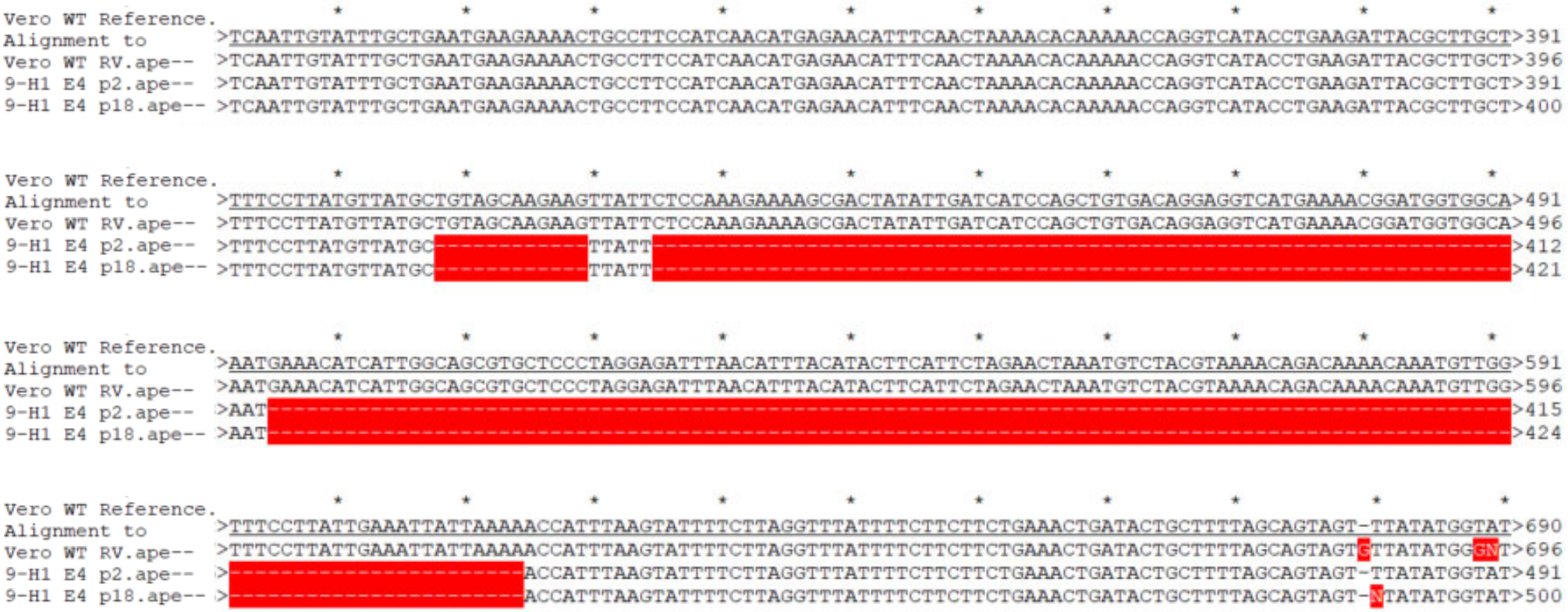


CRISPR/Cas9 edited Vero cells with modified STAT1 gene

Cas9 constructs are expressed transiently and are not integrated into gene edited clones

STAT1 KO in CRISPR-Cas9 Edited Vero Cell Clone

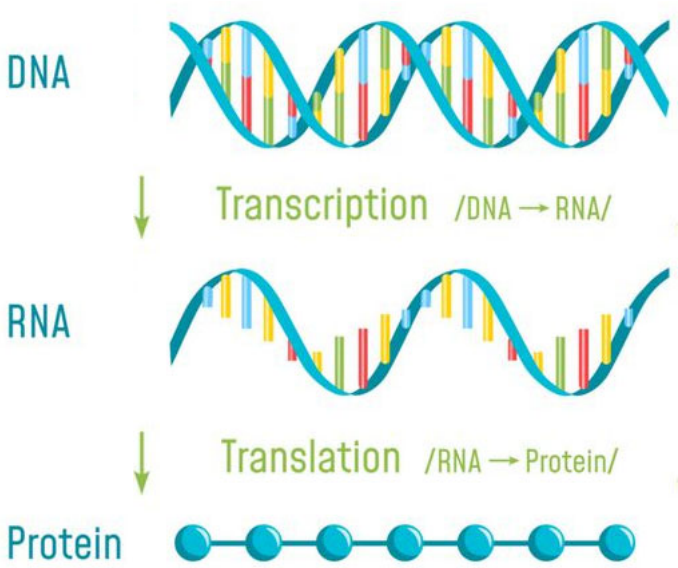
Selected STAT1 KO Vero cell clone has a 199 nt deletion in both chromosomal copies of STAT1



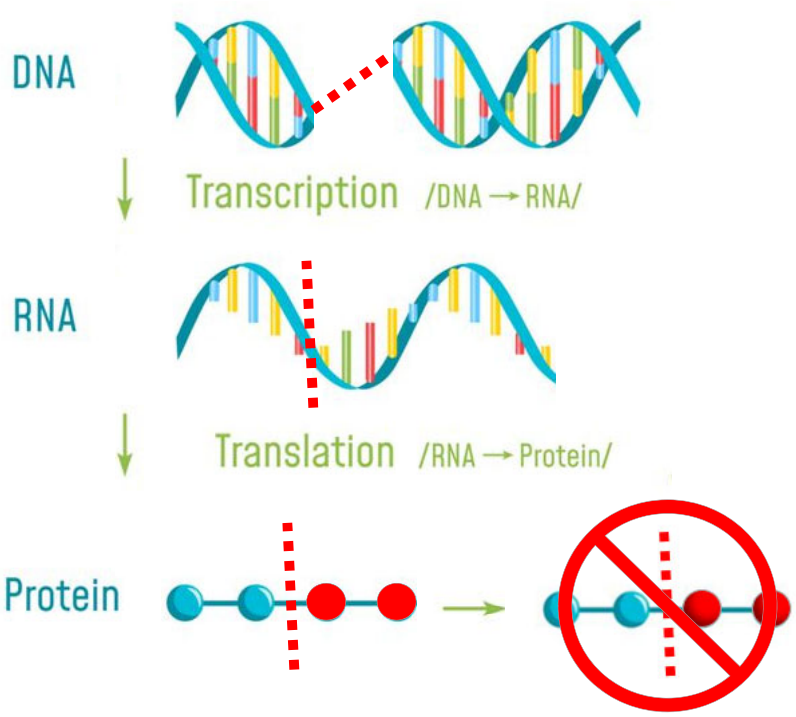
Sequence Deletion Results in Functional STAT1 Knockout

Edited STAT1 gene produces a truncated, non-functional protein that is rapidly degraded

Production of STAT1 Protein in Unmodified Vero Cells

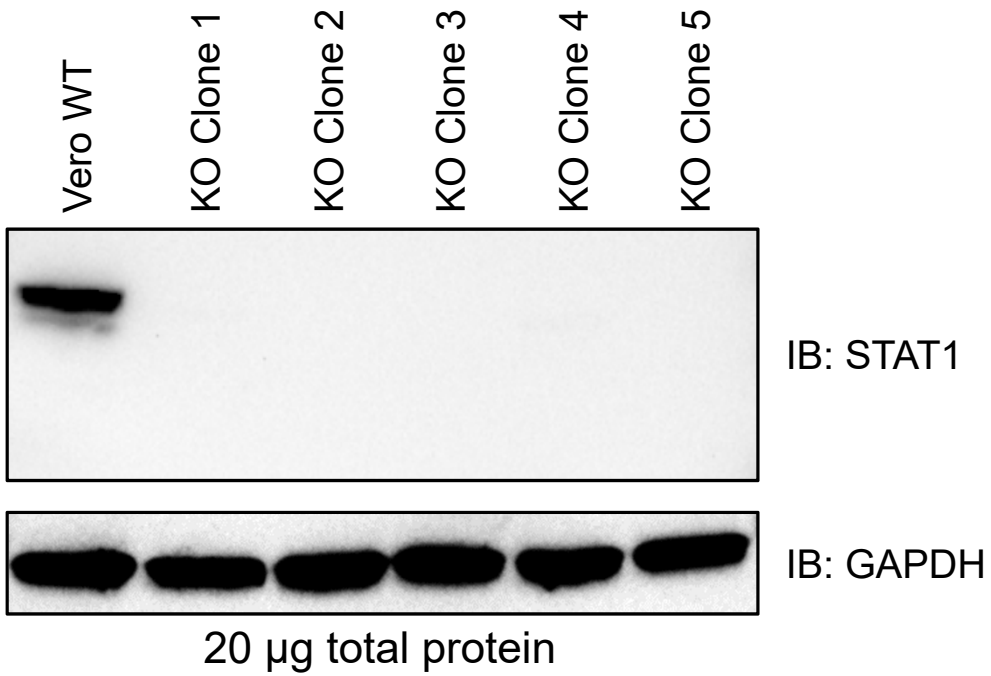


Production of STAT1 Protein in Modified Vero Cells



Confirmation of STAT1 Protein Knockout

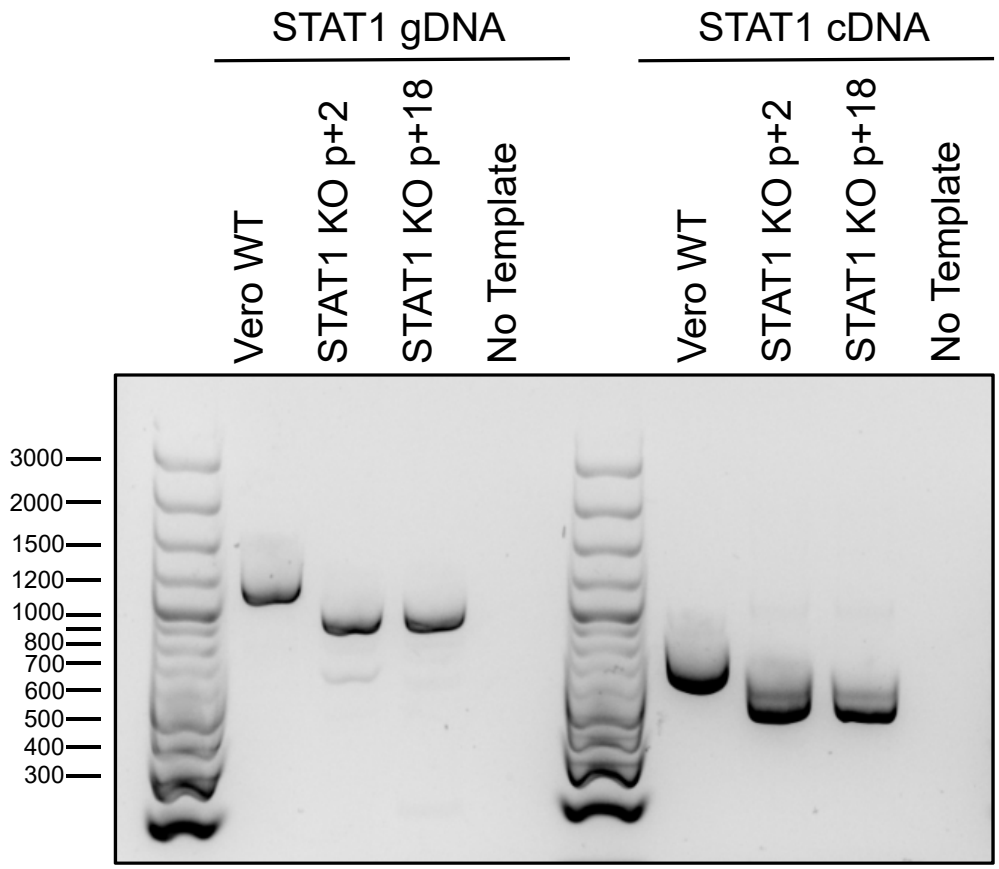
No STAT1 protein detected in selected STAT1 KO Vero clones



Protein Immunoblotting:

- Extract total protein from modified Vero cells
- Separate cellular proteins by size (PAGE gel)
- Transfer separated cellular proteins to blotting paper (PVDF membrane)
- Apply anti-STAT1 antibody to blotting membrane
- Label STAT1 antibody with illuminating dye
- Image to visualize STAT1 protein

Confirmation of STAT1 Gene Disruption

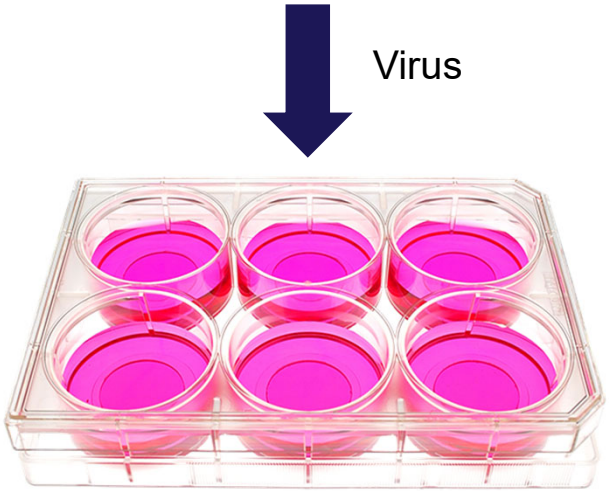


PCR: STAT1

TCID50 Measurement of Virus Concentration

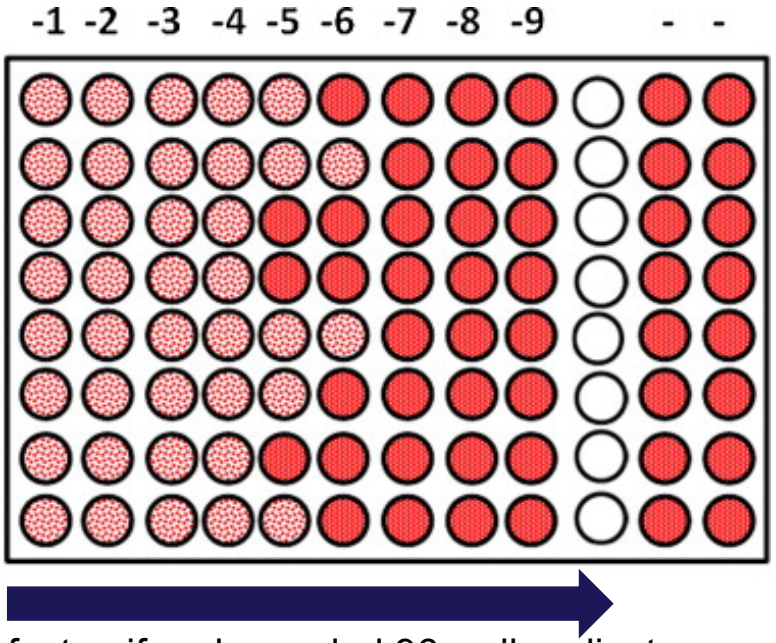
TCID50 – endpoint dilution assay that measures 50% Tissue Culture Infective Dose

1.



Infect WT and STAT1 KO Vero cells with virus of interest, harvest viral supernatants throughout the course of the viral infection.

2.



Infect uniformly seeded 96 well replicate plates of WT Vero cells with serial dilutions of experimental viral supernatants. Score wells as positive or negative for infection. Calculate TCID50.

Journal of Virological Methods. 193(2). 565-571.

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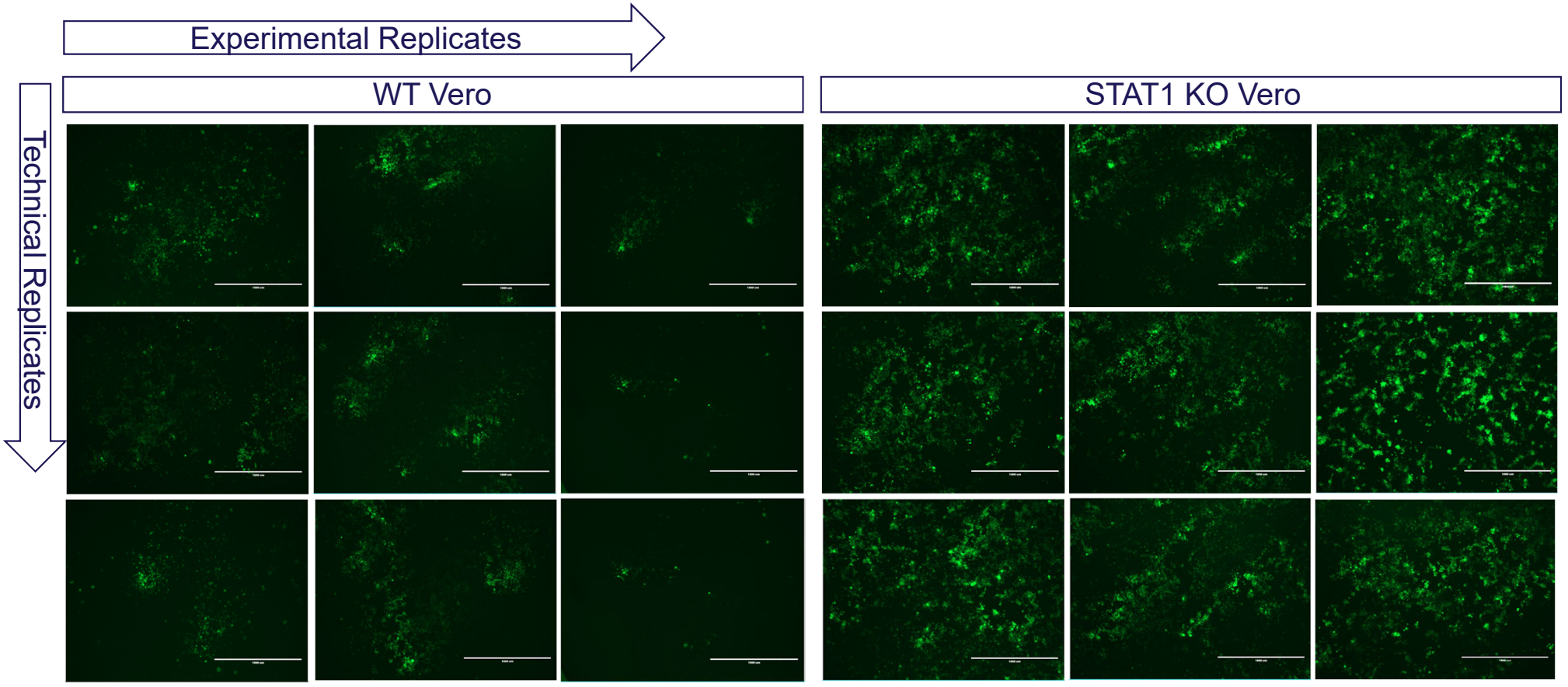
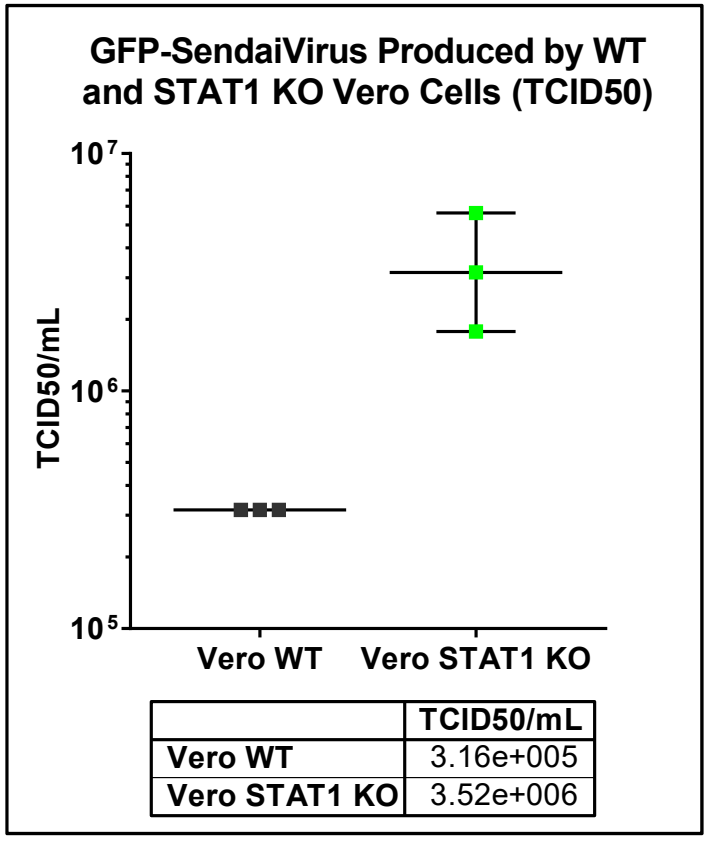
Increased Reporter Virus Production in STAT1 KO Vero

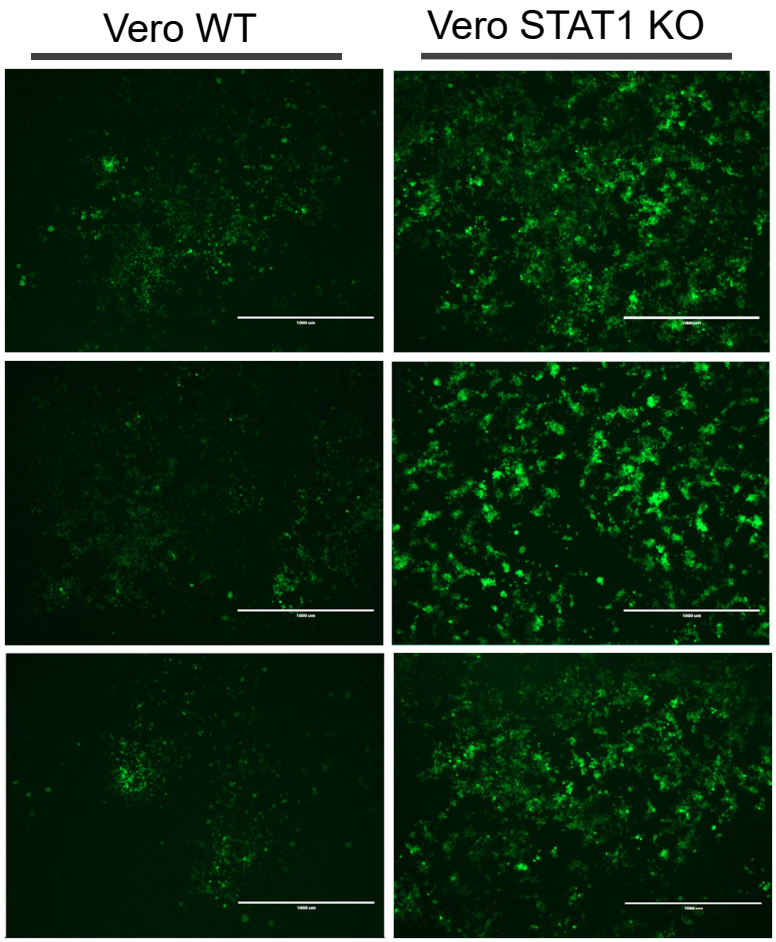


GFP-SeV Production in WT and STAT1 KO Vero Cells

STAT1 KO Vero cells show 10-fold enhancement in GFP-SeV production at 72 HPI

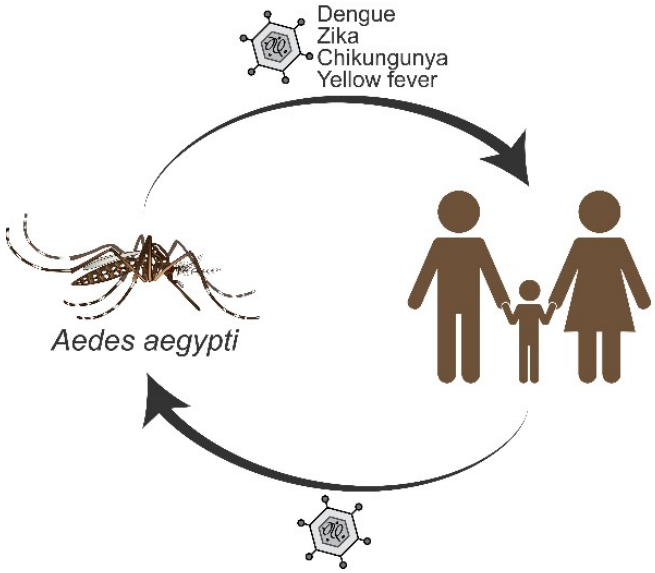


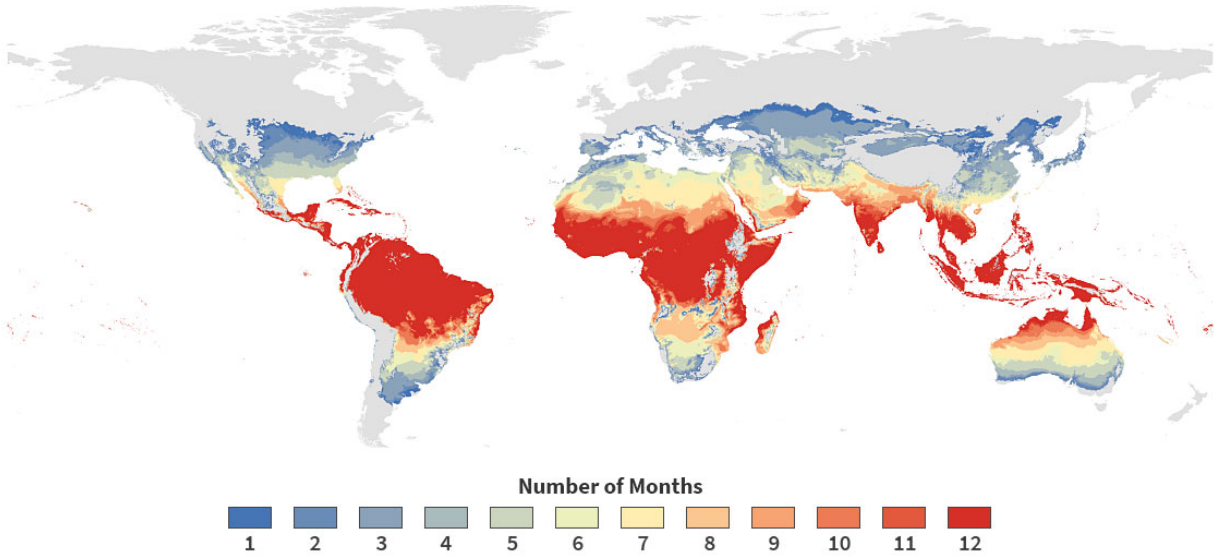
	TCID50/mL
Vero WT	3.16e+005
Vero STAT1 KO	3.52e+006



Vaccines Made in Vero Cells: Dengue Virus

Expansion of Aedes aegypti habitat is increasing the demand for Dengue vaccine





PLoS Negl Trop Dis 13(3): e0007213

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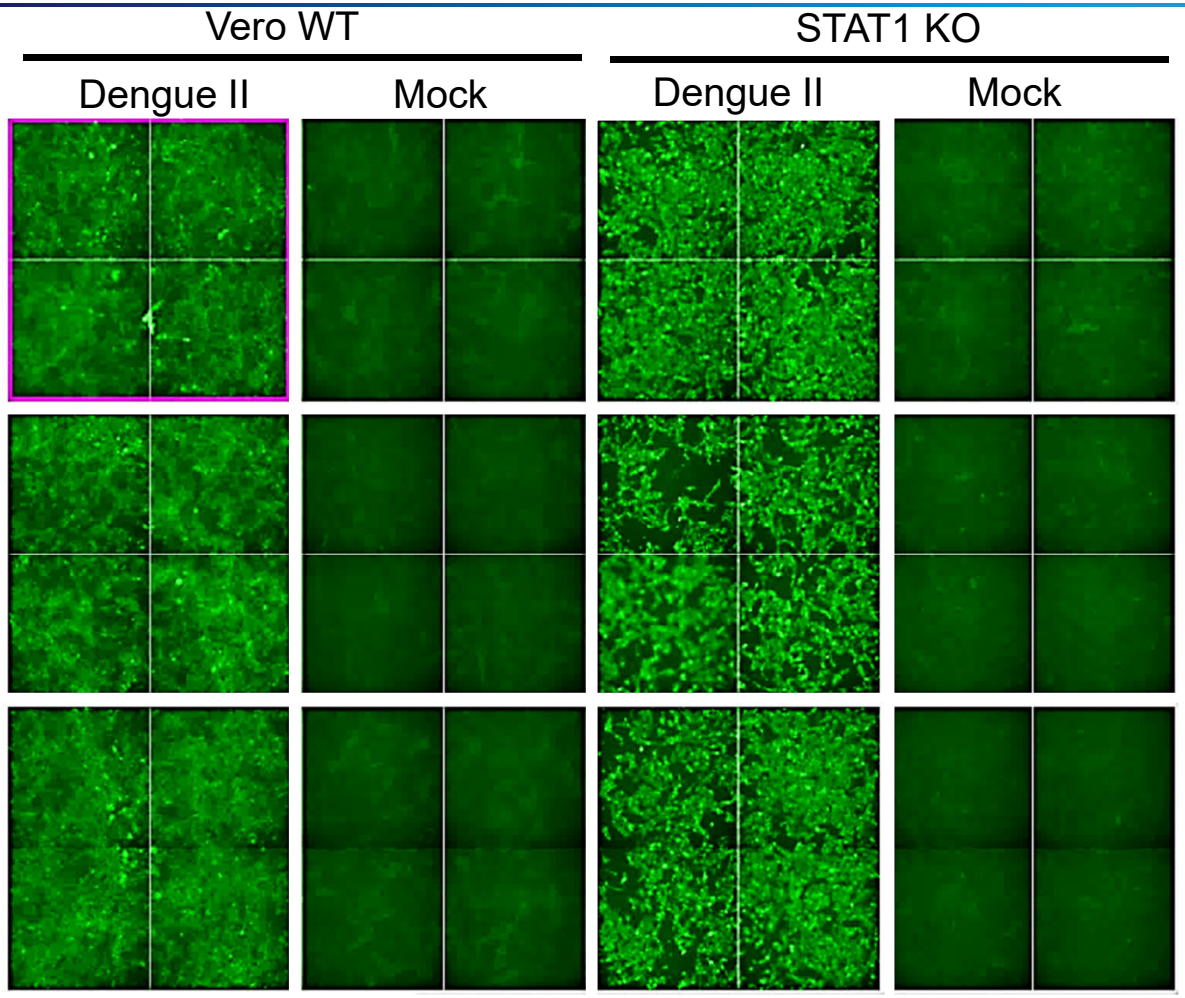
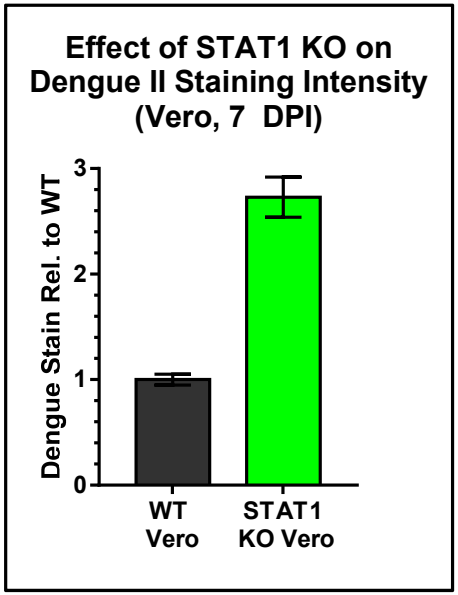
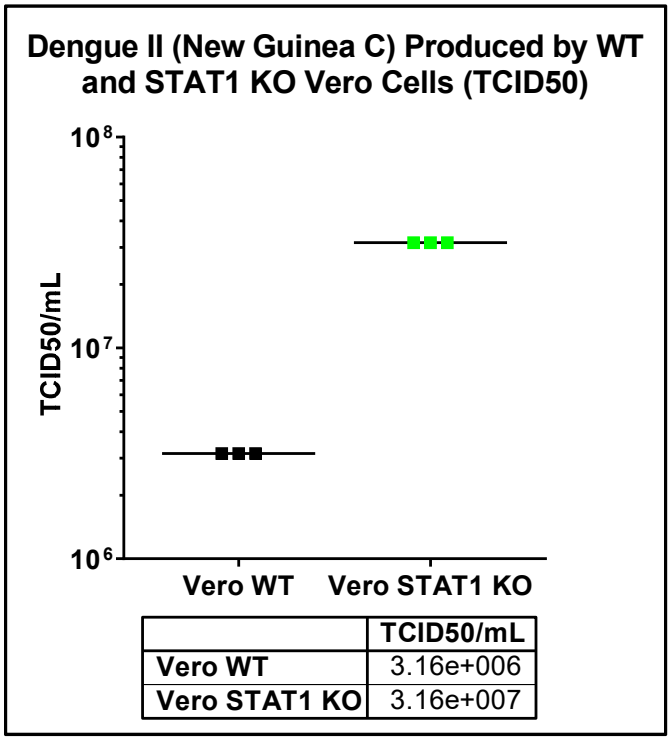
Evaluation of Dengue II Production in STAT1 KO Vero



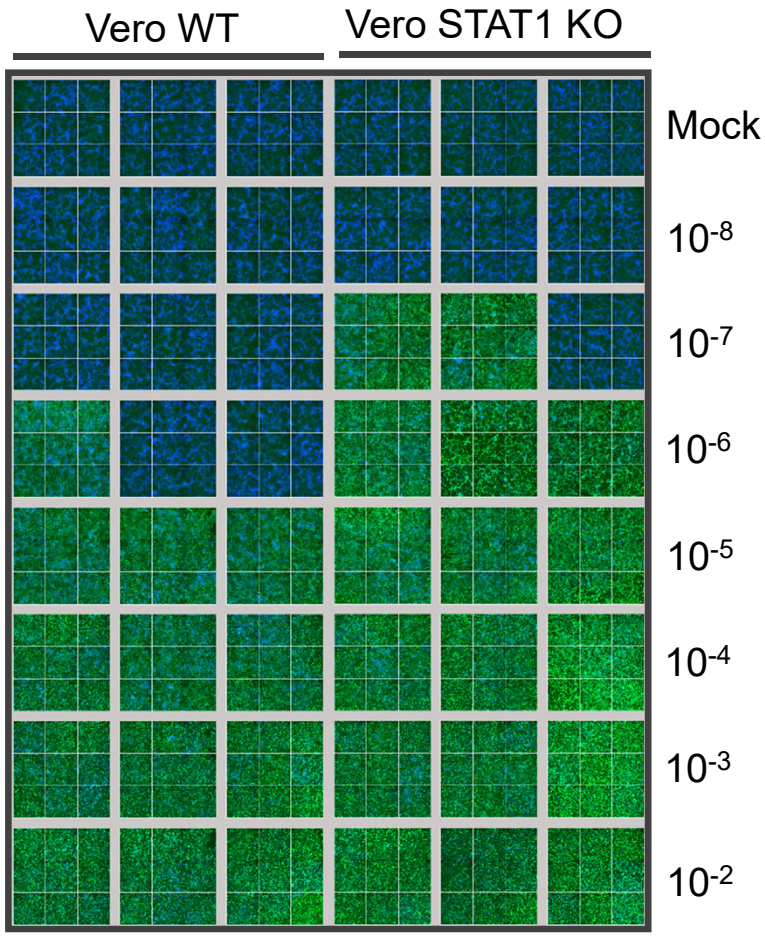


Enhanced Dengue Virus Production – TCID50

Dengue II staining of TCID50 of viral supernatants from WT and STAT1 KO Vero

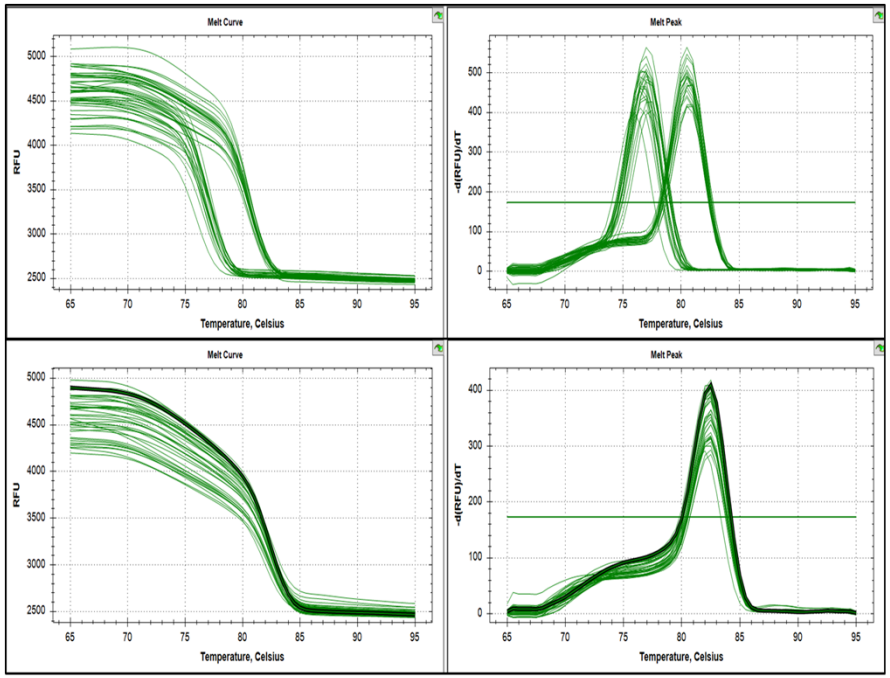


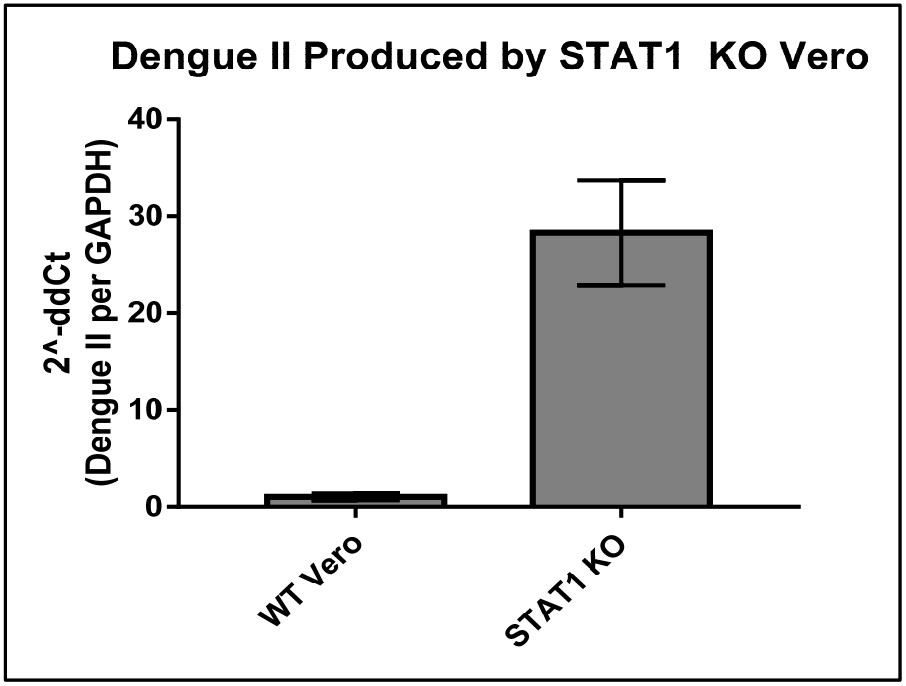
	TCID50/mL
Vero WT	3.16e+006
Vero STAT1 KO	3.16e+007



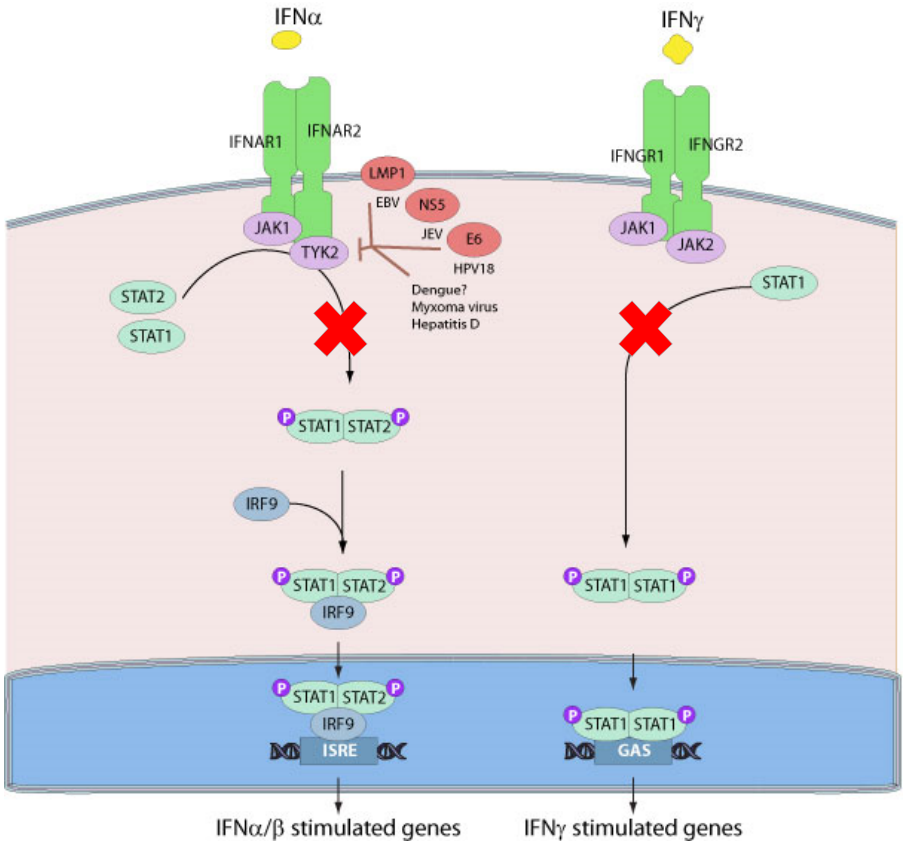
Enhanced Dengue Replication – RT-PCR

RT-PCR shows 30-fold increase in Dengue II viral genomes produced in STAT1 KO Vero cells





STAT1 KO Vero Cells Show No Response to Interferon



	IFNγ	IFNγ
	+	+
WT Vero	WT Vero	KO Vero
+	+	+
GFP-SeV	GFP-SeV	GFP-SeV
⇩	⇩	⇩
Typical GFP-SeV infection	Reduced GFP-SeV infection	Typical GFP-SeV infection

viralzone.expasy.org – inhibition of host STAT1 by virus

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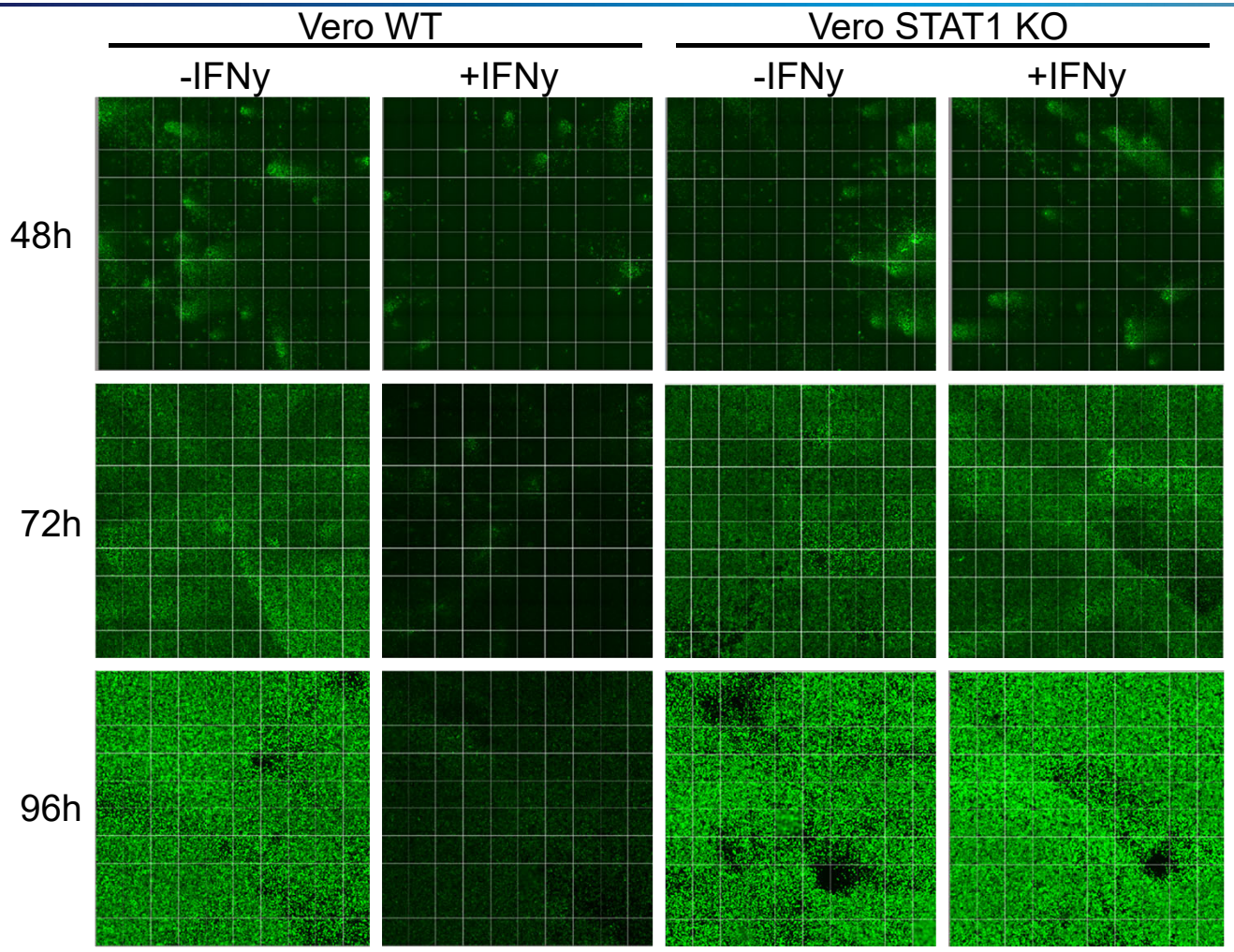
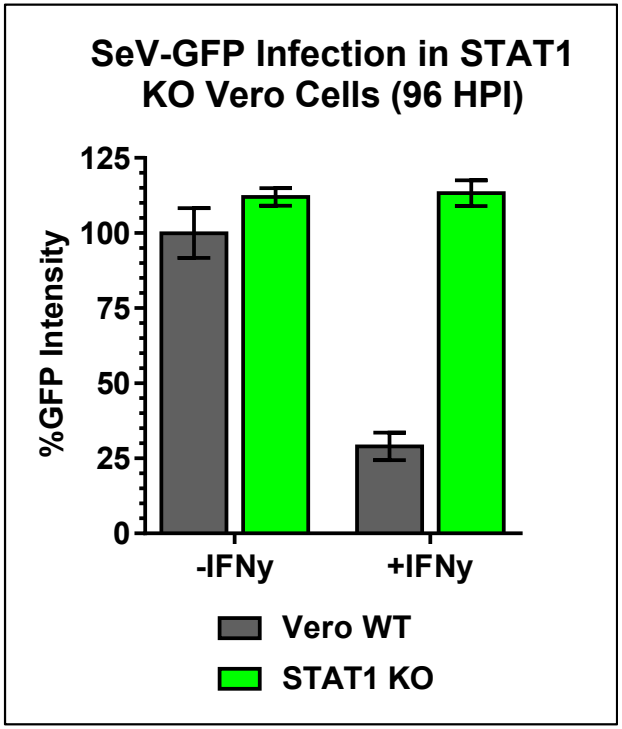
No Response to Interferon in STAT1 KO Vero Cells





Screening for Off-Target Mutations

Cas9 has the potential to create mutations in sequences that resemble the targeted sequence

	Gene	Gene ID	Also Known As	Target Coordinates	Target Sequence	Type	MM	CFD	WT	KO
	STAT1	103217529	Vero G2	chr10:76532444-76532466:-	TCATGACCTCCTGTCACAGC TGG	Exon	0	N/A		
1	**EML6**	103220249	EMAPL6	chr14:52084054-52084076:+	TCAT**C**AC**TG**CCTGTCACAGC CAG	Exon	3	0.1103		
2	**NPB/ PCYT2**	103243738/ 103243739	N/A	chr16:73777880-73777902:+	TC**TGC**ACCTCCTG**C**CACAGC TGG	Exon	4	0.099		
3	**ZNF462**	103219061	ZFPIP, Zfp462	chr12:32612133-32612155:-	TCA**A**G**T**CCTCCT**A**T**A**ACAGC CGG	Exon	4	0.0698		
4	**EPHX1**	103230002	MEH, EPHX, EPOX, HYL1	chr25:3945289-3945311:-	TCA**AC**ACCT**G**CTGTCACA**C**C AGG	Exon	4	0.0652		
5	**SYTL1**	103225294	JFC1, SLP1	chr20:105436957-105436979:-	TC**GG**GACCTCCTG**C**CAC**T**GC GGG	Exon	4	0.0546		
6	**CCDC180**	103219164	C9orf174	chr12:42077653-42077675:+	TCATGACCT**GTG**GTCACAG**A** TGG	Exon	4	0.0299		
7	**TG**	103237469	TGN, AITD3	chr8:127328236-127328258:-	TCATGAC**T**T**A**CT**C**TCACAG**G** CGG	Exon	4	0.0188		
8	**ECH1**	103234640	HPXEL	chr6:33441150-33441172:-	TCAT**C**ACC**G**CCTGT**G**ACA**T**C CGG	Exon	4	0.0162		
9	**GSDMA**	103243525	GSDM, FKSG9, GSDM1	chr16:66196251-66196273:+	**C**CA**C**GACCTCCT**T**T**G**ACAGC AGG	Exon	4	0.0083		
10	**NRXN2**	103233117	N/A	chr1:9583866-9583888:-	TCATG**G**CCTCC**CA**TCACAGC TGG	Intron	3	0.6246		
11	**ASIC4**	103217917	ACCN4, BNAC4	ch10:105414648-105414670:-	**G**CATGAC**T**TCC**AA**TCACAGC CGG	Intron	4	0.6181		
12	**FHOD3**	103222526	FHOS2, Formactin2	chr18:44299974-44299996:-	**C**CA**G**GACCT**A**CTG**A**CACAGC AGG	Intron	4	0.2874		

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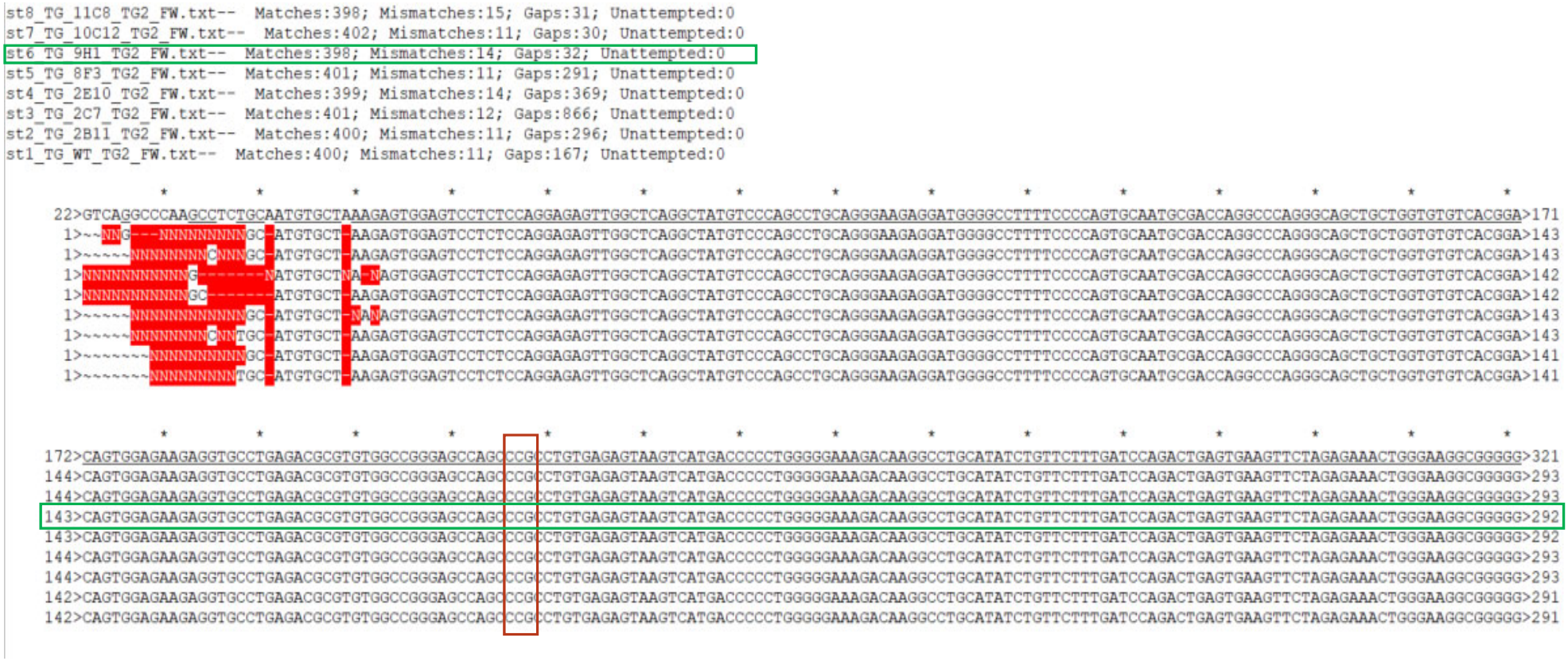
Example of Off-Target Mutation Evaluation

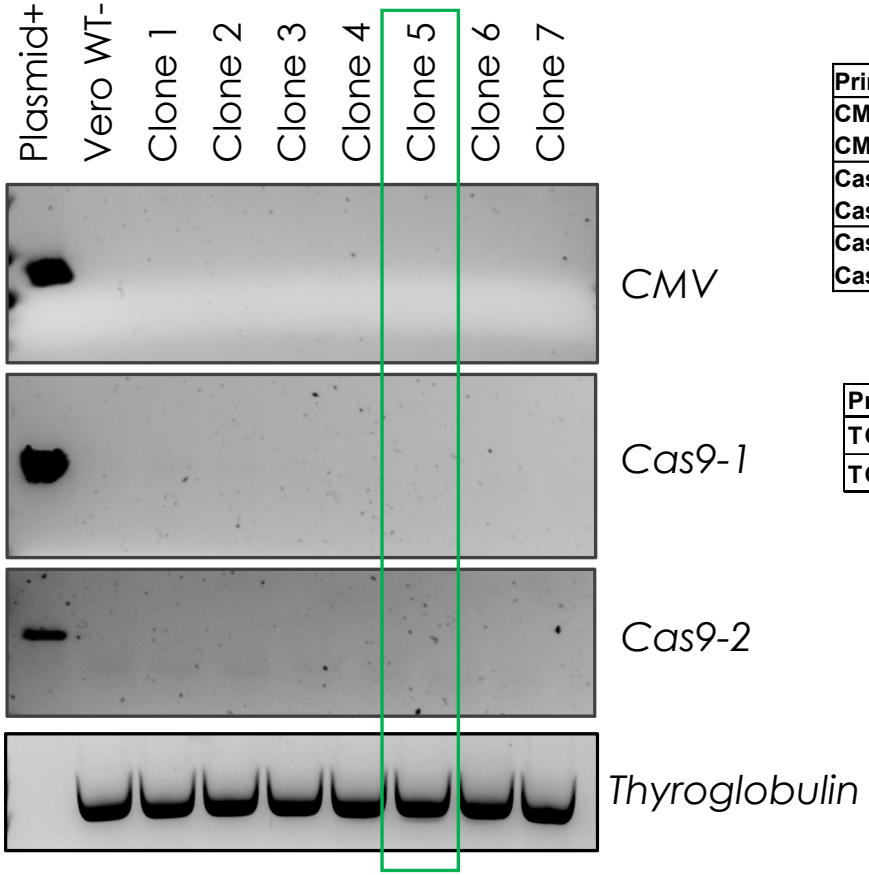
Sanger sequencing of off-target site shows no mutation across multiple gene-edited clones



Off-target cut site location

Cas9/CMV Plasmid Integration Assessment

No Cas9/CMV integration in selected STAT1 KO Vero clone



Primer	Sequence (5' to 3')	Tm (Q5)	Anneal	Product
CMV-FW	TGGCTCTAGAGGTACCCGTTACATAAC	69	70	342
CMV-RV	AGATGGGGAGAGTGAAGCAGAAC	69		
Cas9-1-FW	CTATAAGGACCACGACGGAGACTACAAG	69	70	323
Cas9-1-RV	TTCTTCTGGCGGTTCTCTTCAGC	69		
Cas9-2-FW	TGTCTGCCAGACTGAGCAAGAG	69	70	300
Cas9-2-RV	TCTCGGTGTTCACTCTCAGGATGT	69		

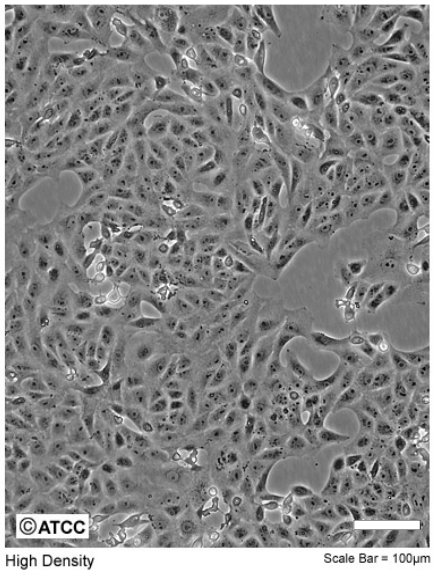
Primer	Sequence (5' to 3')	Tm (Q5)	Anneal	Product
TG FW	GCTCATCTGGCTTGTCTCTGTGT	69˚C	70˚C	**441 bp**
TG RV	CCCAGGCTCTTTCTGACTTCAGTTC	69˚C		

Selected Clone

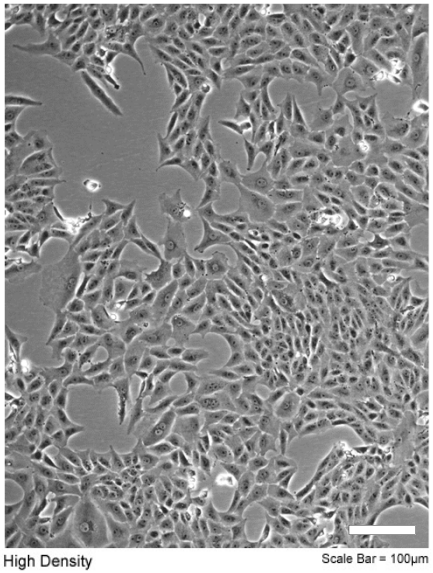
Morphology and Growth Kinetics of STAT1 KO Vero Cells

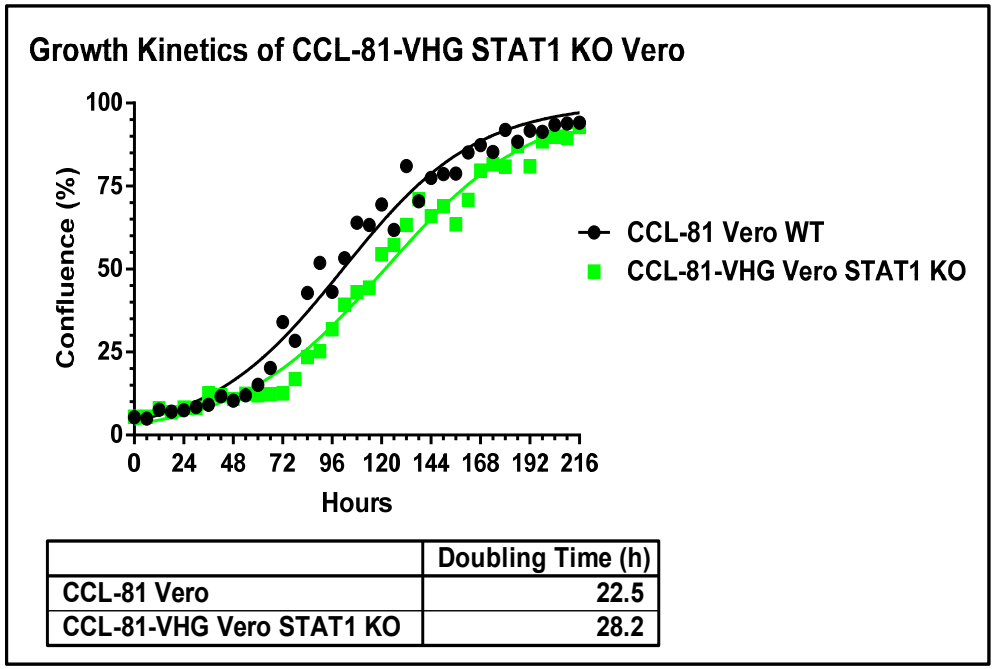
Modified cell line has similar morphology and grows slower than parental Vero cell line

WT Vero



STAT1 KO Vero

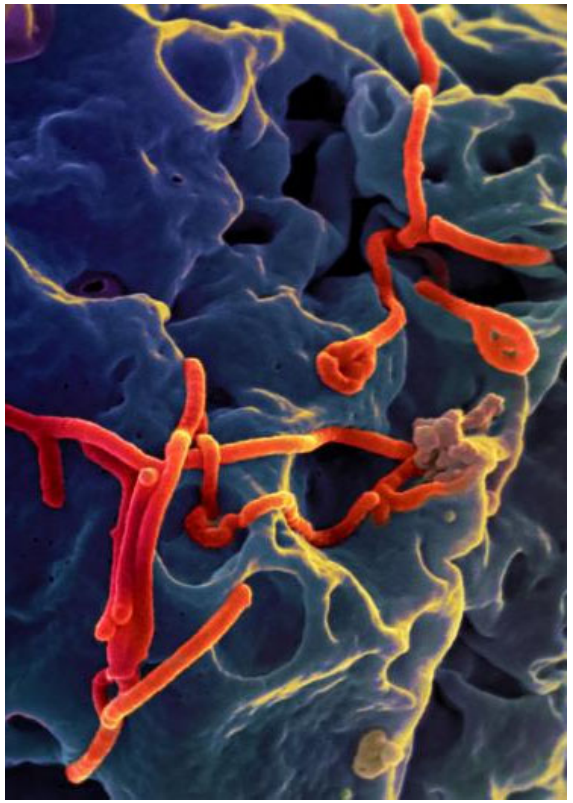




	Doubling Time (h)
CCL-81 Vero	22.5
CCL-81-VHG Vero STAT1 KO	28.2

Summary: Enhanced Viral Production Vero Cells

- Successful STAT1 knockout in Vero cells using CRISPR/Cas9
- 10-fold higher yield of both reporter virus and model clinical virus (Dengue II) in STAT1 KO Vero cells relative to unmodified cells
- 30-fold increase in Dengue viral genome replication in STAT1 KO Vero cells
- STAT1 KO Vero cells show no anti-viral response to interferon treatment
- STAT1 knockout genotype is stable over time in selected Vero clone
- No Cas9 plasmid integration detected in selected STAT1 KO Vero clone
- No mutations detected at selected Cas9 off-target cut sites
- STAT1 KO Vero cells have similar morphology and a slower doubling time than unmodified Vero cells



Ebola virus budding from a Vero cell, image courtesy of NIAID

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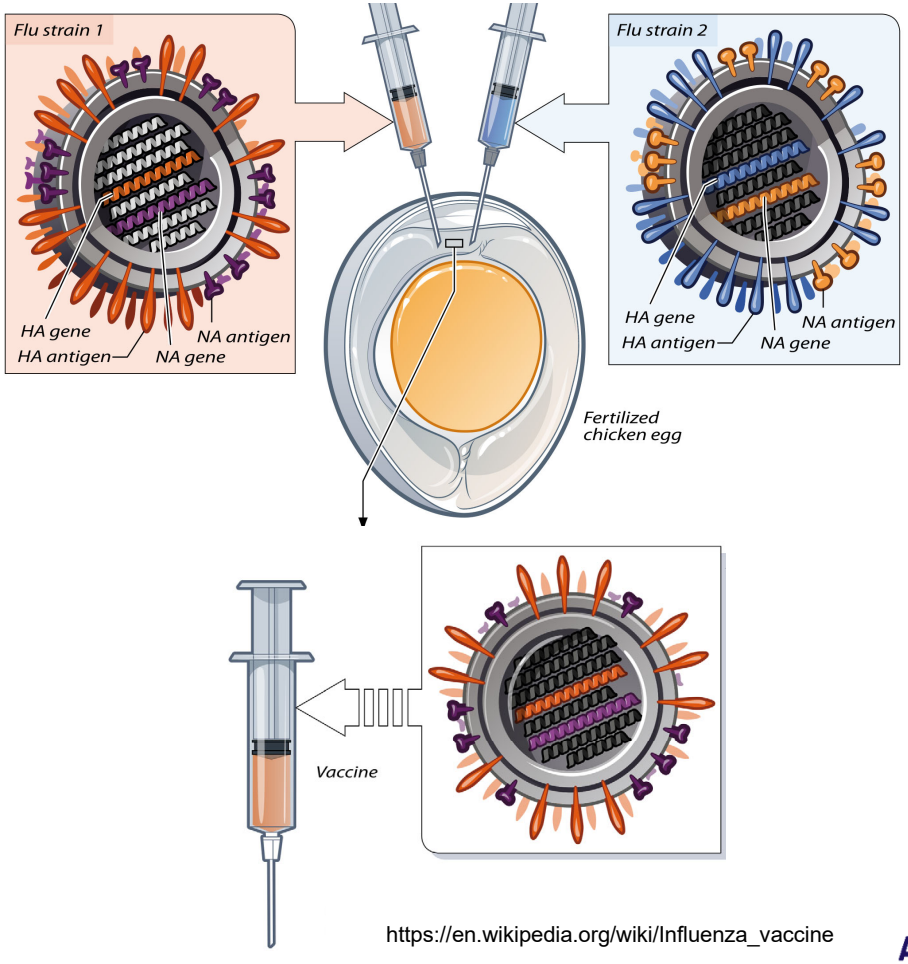
MDCK Cells for Influenza Vaccine Production

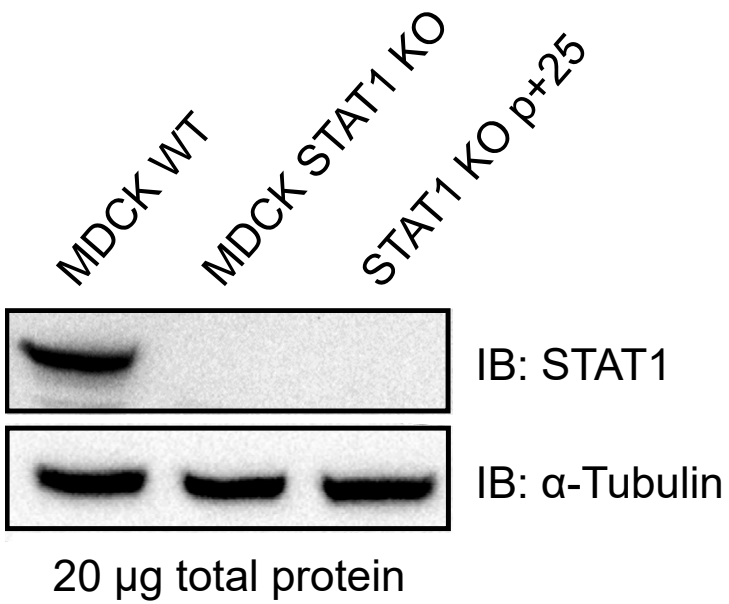
- Over 20% of the human population is infected with the influenza virus each year.
- Traditional production of flu vaccines involves infection and subsequent harvest of embryonated chicken eggs.
  - Time constraints, high production cost
  - High risk of microbial contamination
  - Allergic reactions in some recipients
- Mammalian cell culture-based Influenza vaccines as alternatives to egg-based vaccines.
- A need for rapid and large-scale production of flu vaccine for pandemic preparedness.
- 2012, FDA approved Flucelvax as the first mammalian cell-based (MDCK-produced) Influenza vaccine in US.



https://en.wikipedia.org/wiki/Influenza_vaccine

STAT1 Protein Knockout in MDCK Cells

Stability of STAT1 KO MDCK clone confirmed by immunoblot

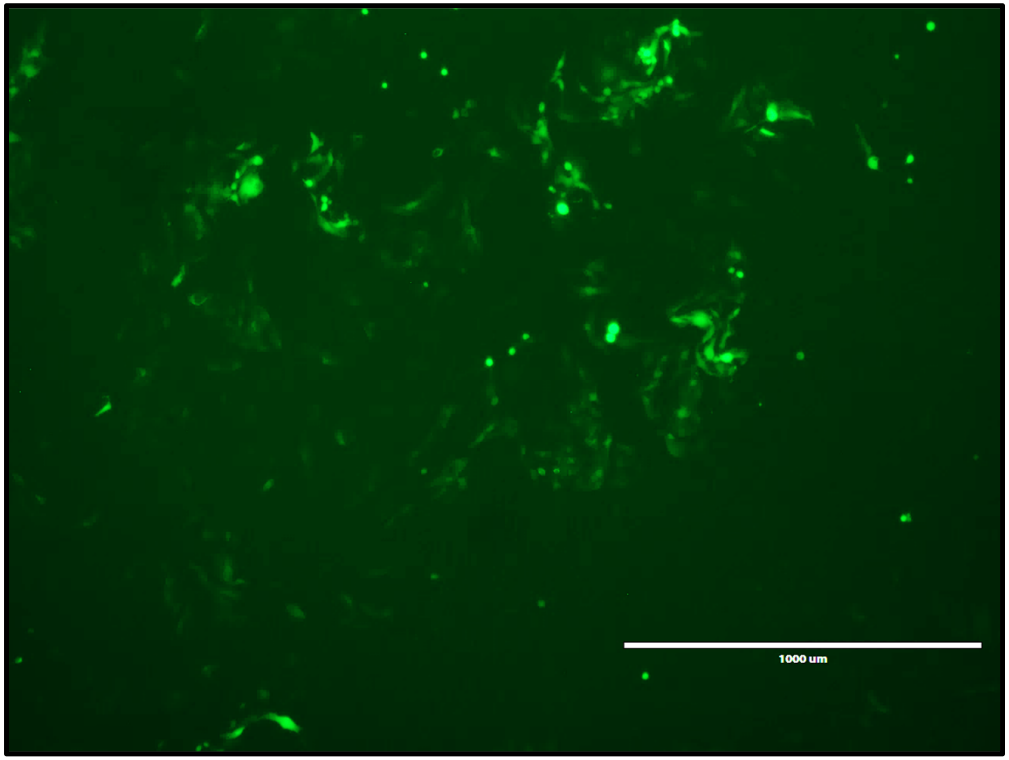


- Short nucleotide insertions and deletions near the beginning of the STAT1 gene were made using CRISPR/Cas9.
- The modified STAT1 gene produces truncated, non-functional STAT1 protein.
- Truncated STAT1 protein is rapidly degraded, resulting in a functional STAT1 protein knockout.
- Lack of functional STAT1 protein results in a lack of anti-viral interferon signaling.

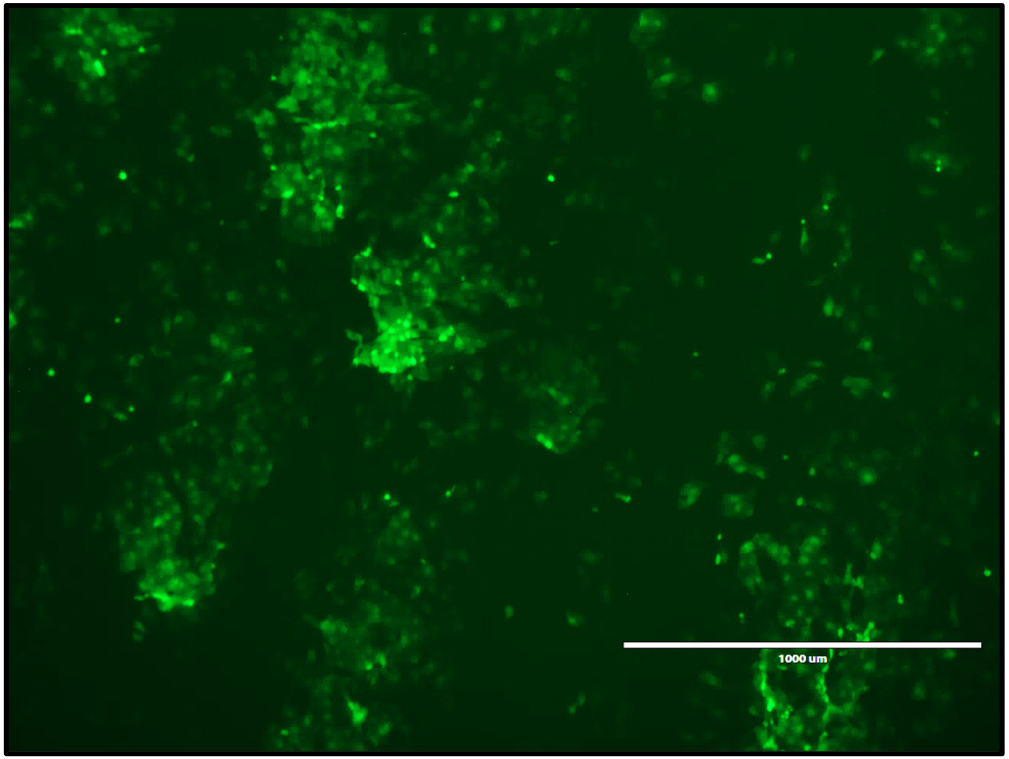
GFP-SeV Infection in WT and STAT1 KO MDCK Cells

GFP reporter virus replicates more quickly in STAT1 KO MDCK cells than in WT MDCK cells

MDCK WT + GFP-SeV (48 HPI)



MDCK STAT1 KO + GFP-SeV (48 HPI)



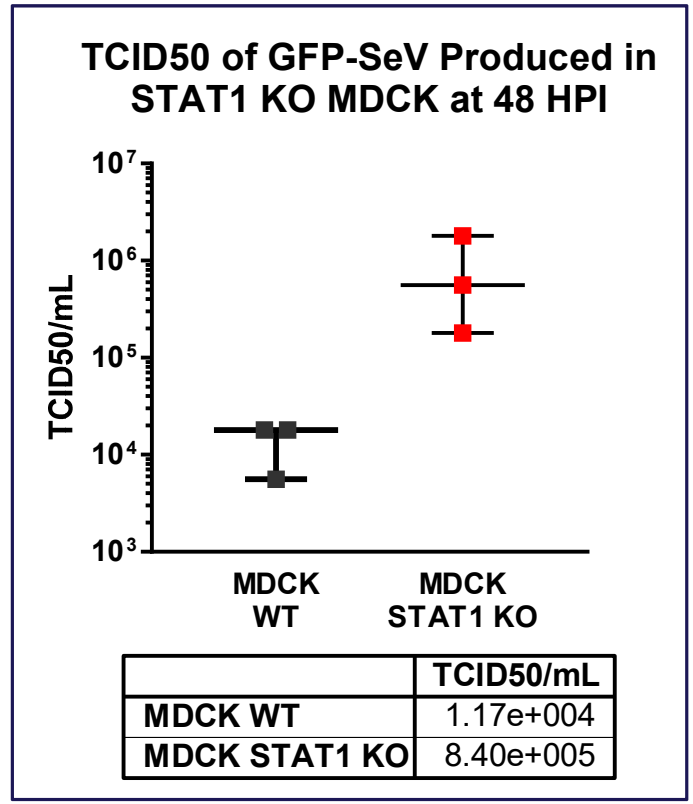
GFP-SeV Production in WT and STAT1 KO MDCK Cells

>50-fold increase in GFP-SeV production in STAT1 KO MDCK

Technical Replicates

Dilution	MDCK WT			MDCK STAT1 KO		
-7	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
-6	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
-5	-	-	-	-	-	+
	-	-	-	-	-	-
	-	-	-	-	+	+
-4	-	-	-	+	+	+
	-	-	-	+	+	+
	-	-	-	-	+	+
-3	+	+	+	+	+	+
	-	-	+	+	+	+
	+	-	-	+	+	+
-2	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
TCID50/mL	1.78E+04	5.62E+03	1.78E+04	1.78E+05	5.62E+05	1.78E+06
Average	1.37E+04			8.40E+05		

Experimental Replicates



	TCID50/mL
MDCK WT	1.17e+004
MDCK STAT1 KO	8.40e+005

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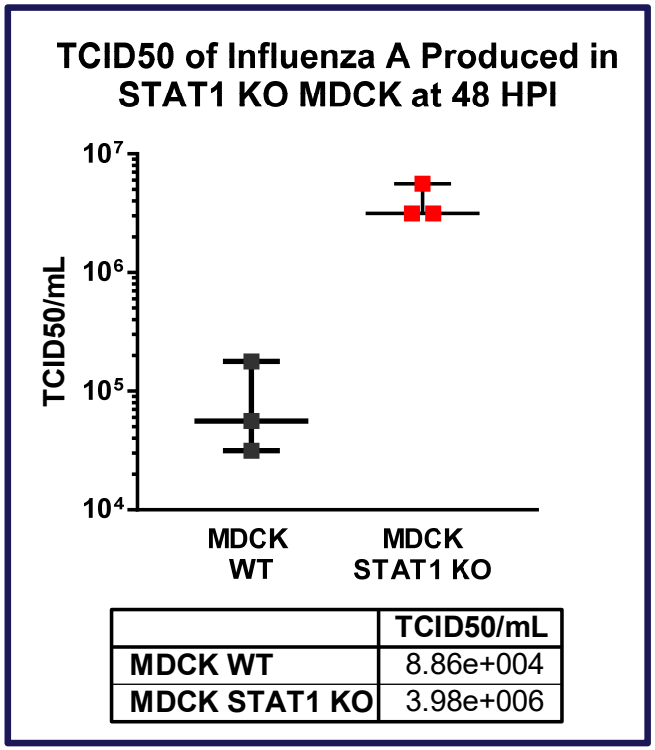
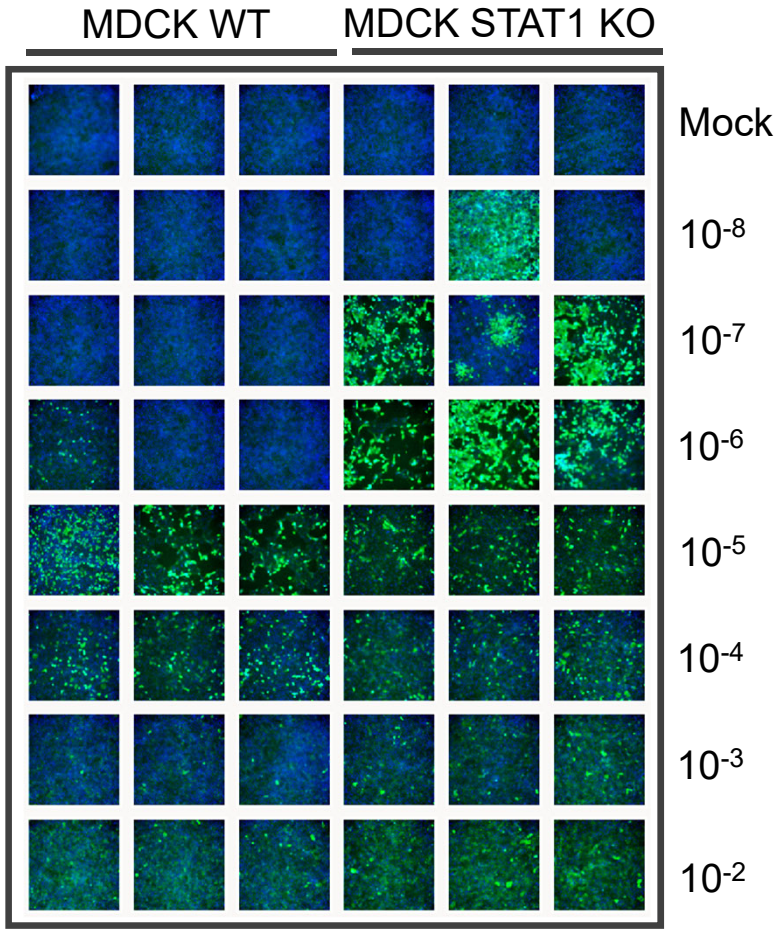
Production of Influenza A in STAT1 KO MDCK

Staining of TCID50 of viral supernatants from STAT1 KO MDCK shows 30-fold increase in viral production

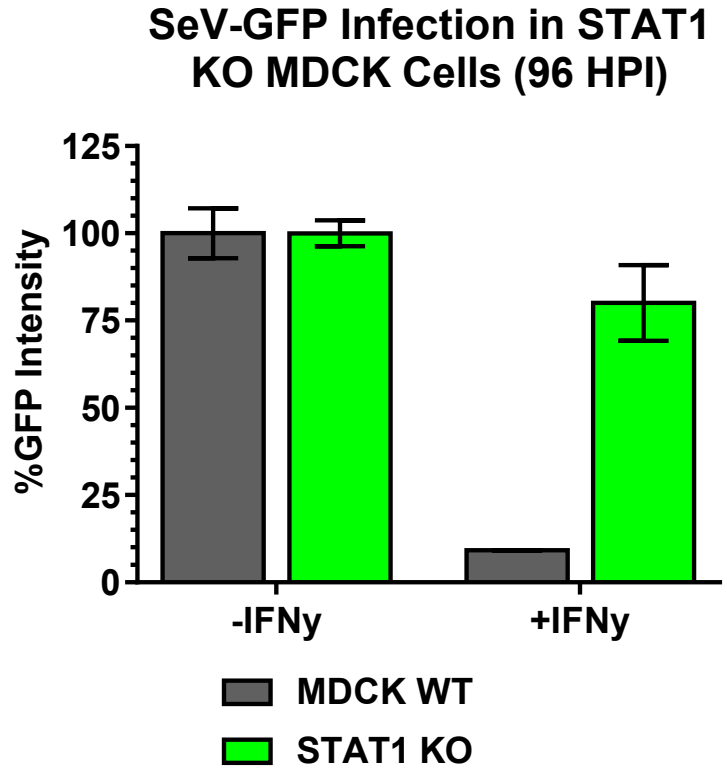


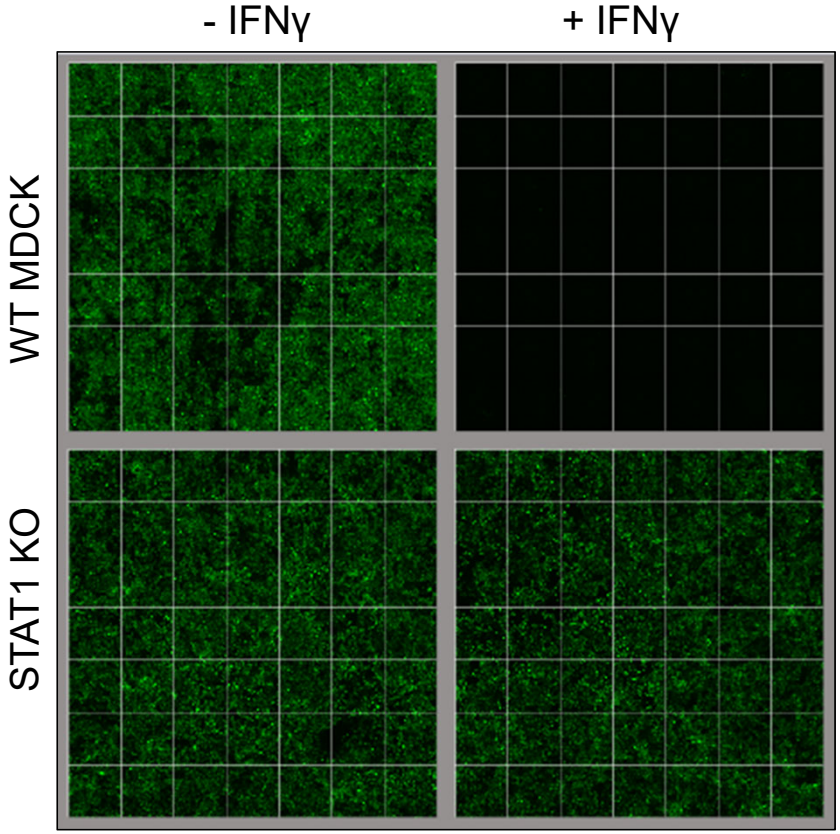
	TCID50/mL
MDCK WT	8.86e+004
MDCK STAT1 KO	3.98e+006



No Response to Interferon in STAT1 KO MDCK Cells

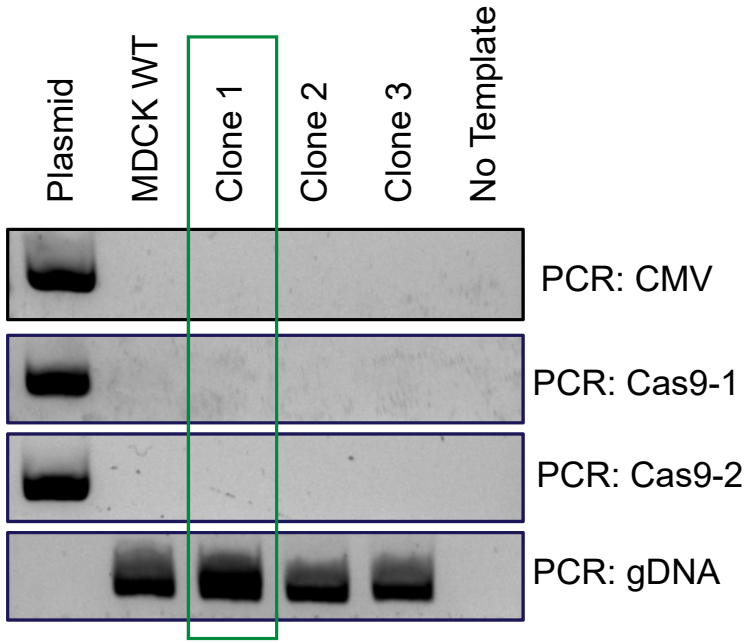
MDCK cells were treated with recombinant canine interferon gamma then infected with reporter virus





Cas9/CMV Plasmid Integration Assessment

No Cas9/CMV integration in STAT1 KO MDCK clone



Primer	Sequence (5' to 3')	Tm (Q5)	Anneal	Product
CMV-FW	TGGCTCTAGAGGTACCCGTTACATAAC	69	70	342
CMV-RV	AGATGGGGAGAGTGAAGCAGAAC	69		
Cas9-1-FW	CTATAAGGACCACGACGGAGACTACAAG	69	70	323
Cas9-1-RV	TTCTTCTGGCGGTTCTCTTCAGC	69		
Cas9-2-FW	TGTCTGCCAGACTGAGCAAGAG	69	70	300
Cas9-2-RV	TCTCGGTGTTCACTCTCAGGATGT	69		
cfSTAT1 E2 FW3	GGCTTCTTGAATAATTTTCATAAGGAAAGCA	65	66	247
cfSTAT1 E2 RV3	CTTATGCTTGGGAACATTTTGGC	65		

Selected Clone

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Screening for Off-Target Mutations in STAT1 KO MDCK

No mutation detected in Cas9 off-target screen

	Gene	Gene ID	Gene Description	Target Sequence	Type	MM	CFD	Tm (Q5)	Anneal:	Product	STAT1 KO
	STAT1	488449	signal transducer and activator of transcription 1	GAGGTCGTGAAAACGGATGG TGG	Exon	0	N/A	65˚C 65˚C	66˚C	247 bp	
1	**CDH13**	489689	cadherin 13	GA**A**GTC**A**TGAAAA**T**GGATG**A** AGG	Intron	4	0.516	66˚C 68˚C	67˚C	331 bp	
2	**MYRIP**	485603	myosin VIIA and Rab interacting protein	GAGGTC**CA**GAAAAC**A**GATG**A** GGG	Intron	4	0.485	67˚C 68˚C	68˚C	442 bp	
3	**ASIC2**	491150	acid sensing ion channel subunit 2	GAGGTC**AG**GAAA**G**C**A**GATGG GGG	Intron	4	0.450	68˚C 67˚C	68˚C	413 bp	
4	**CCDC170**	476248	coiled-coil domain containing 170	**AA**AGT**TC**TGAAAACGGATGG TGG	Intron	4	0.431	68˚C 68˚C	69˚C	618 bp	
5	**PAK5**	485772	p21 (RAC1) activated kinase 5	**A**AGGTC**C**TGAA**G**A**T**GGATGG AGG	Intron	4	0.328	67˚C 67˚C	68˚C	537 bp	
6	**SLAMF6**	100684044	SLAM family member 6	G**G**GG**G**CGTG**G**AAAC**A**GATGG GGG	Intron	4	0.268	69˚C 67˚C	68˚C	795 bp	

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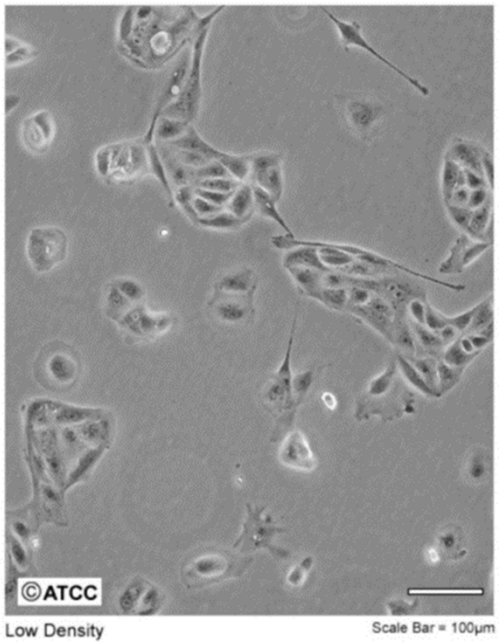
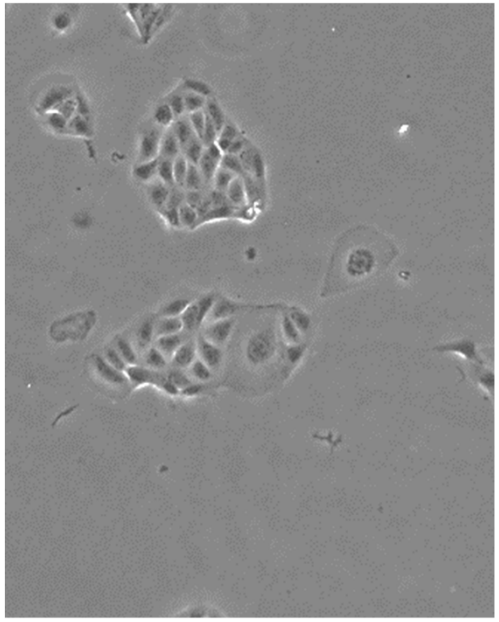
MDCK Morphology and Growth Kinetics

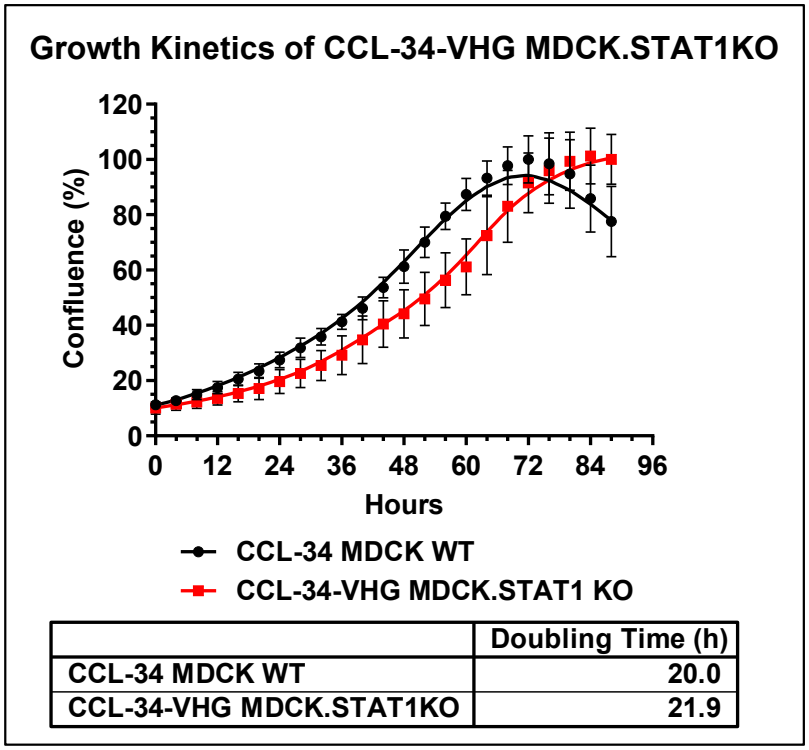
Modified cell line has similar morphology and grows slightly slower than parental MDCK cell line



CCL-34 MDCK



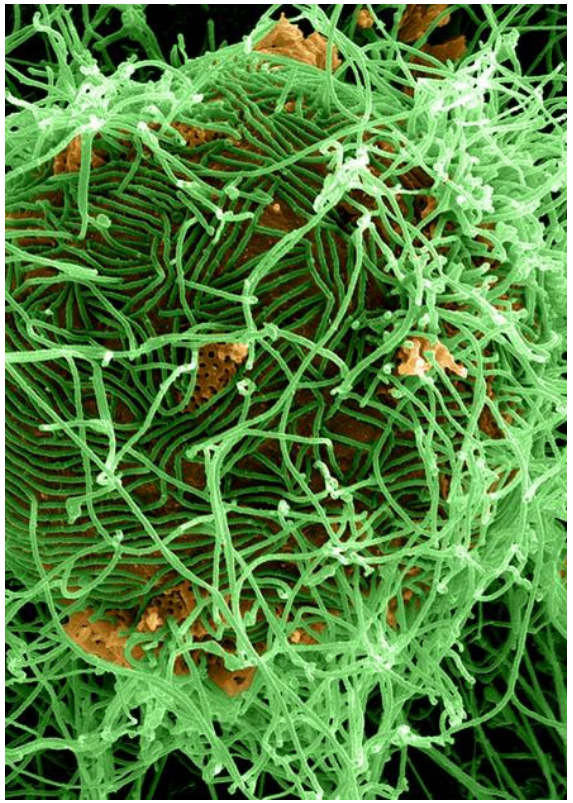
CCL-34-VHG MDCK.STAT1KO



	Doubling Time (h)
CCL-34 MDCK WT	20.0
CCL-34-VHG MDCK.STAT1KO	21.9

Summary - Enhanced Viral Host Cells

- CRISP-Cas9 is a powerful technology for precise genetic modification in various organisms. ATCC is broadly utilizing CRISPR across our portfolio of cell lines to develop enhanced products to meet critical customer needs.
- Vero and MDCK cells have been modified by CRISPR-Cas9 to have a permanent increase in viral production capacity.
- Modified Vero and MDCK cells have been thoroughly characterized for viral production phenotype, stability of STAT1 knockout genotype, lack of Cas9 plasmid integration, and absence of off-target mutations.
- Enhanced viral production cell lines have broad applicability for basic research, production of high-titer viral stocks, and manufacturing of viruses and viral vaccines.



Ebola virus infecting Vero cell, image courtesy of NIAID

For more information visit www.atcc.org/vaccinedev

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ATCC Coronavirus Research Materials

- Synthetic Molecular Standards for SARS-CoV-2 ***Coming Soon!***
- Coronavirus strains:
  - Heat-inactivated SARS-CoV-2 ***Coming Soon!***
  - Betacoronavirus 1 OC43
  - Human coronavirus 229E
- SARS-CoV-2 Genomic RNA ***Coming Soon!***
- Cell Lines for Enhanced Virus Production
- Custom Solutions

www.atcc.org/coronavirus