Reproducibility of indel formation rates by comparing guideRNA format and delivery method

GERG Study 2018-2019

ABRF 2019 Annual Meeting

Kym Delventhal

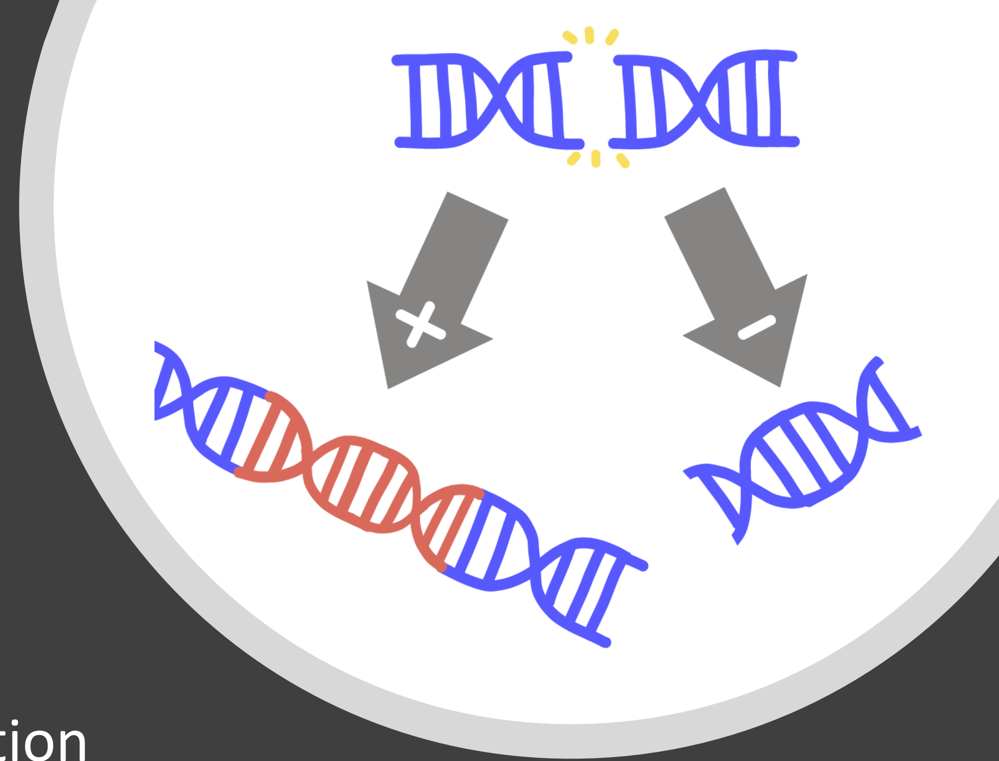
Stowers Institute for Medical Research

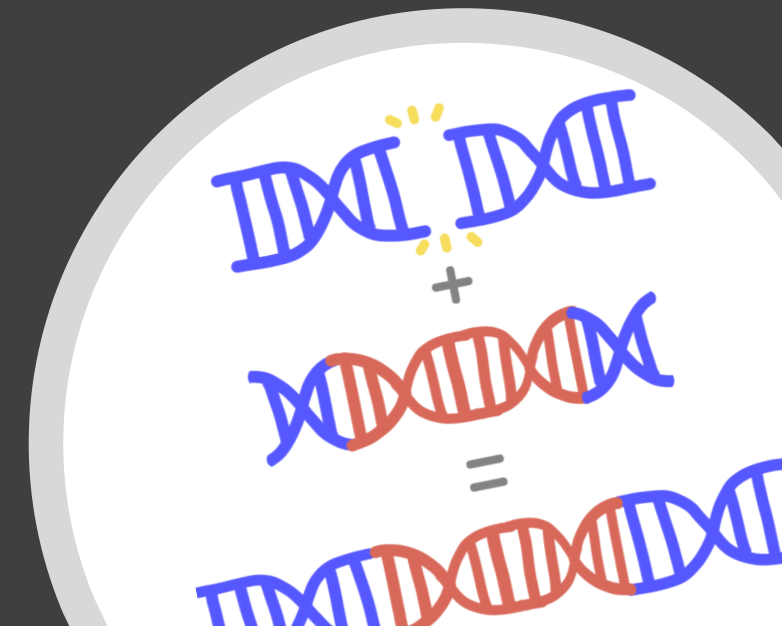
GERG

- Started in 2015 with 4 members
- Currently have 8 members
  - Kym Delventhal (Co-Chair) Stowers Institute
  - Elizabeth Sergison (Co-Chair) Dartmouth College
  - Shondra M. Pruett-Miller - St. Jude Children's Research Hospital
  - Channabasavaiah Gurumurthy - University of Nebraska Medical Center
  - Eric Kmiec - Gene Editing Institute
  - Maureen Regan – University of Illinois Chicago
  - Timothy J. Dahlem - Recursion Pharmaceuticals
  - Gerald Marsischky – Independent Consultant (not pictured)

Genome Engineering

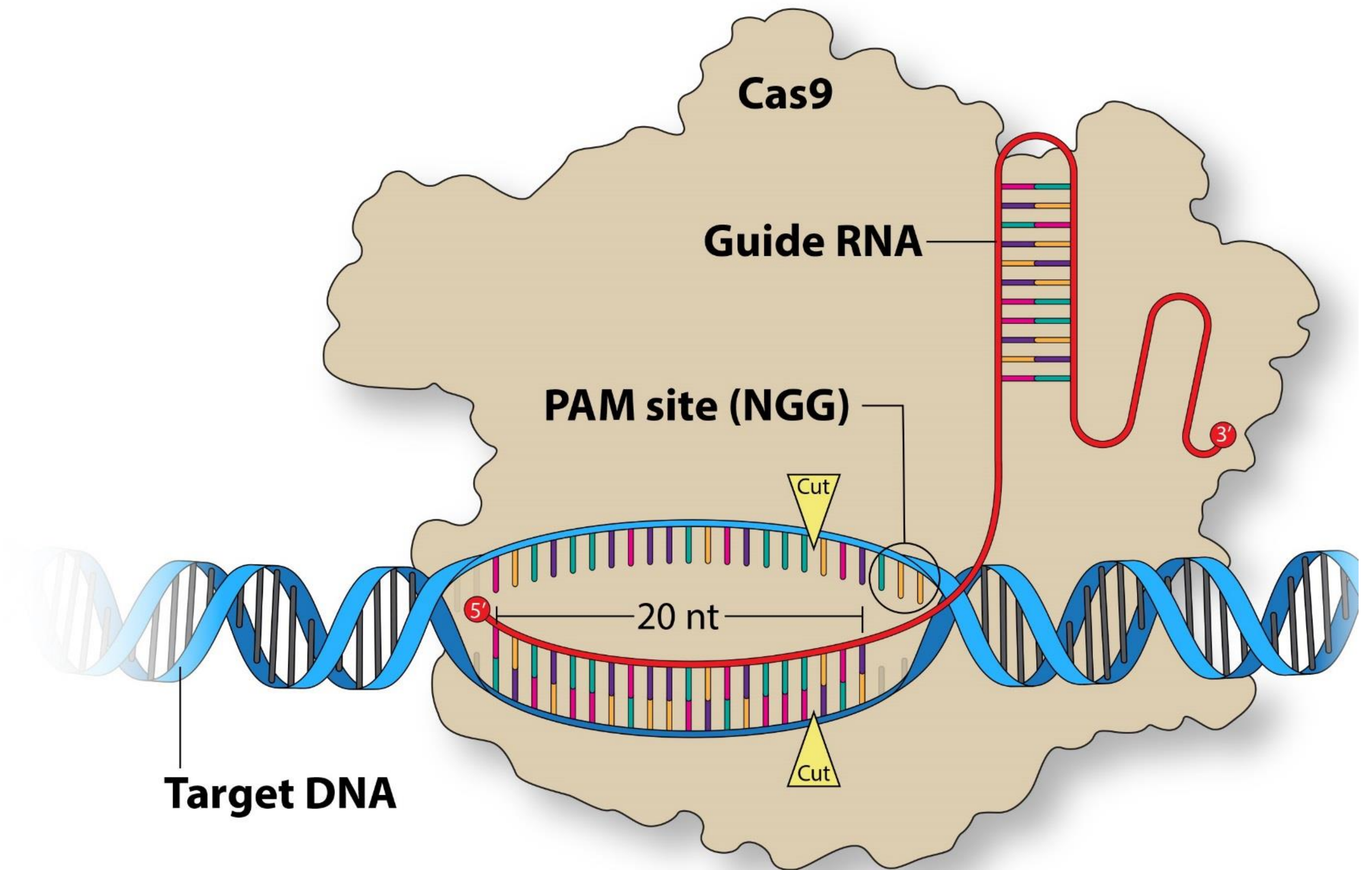
- Allows for targeted modifications of genomic DNA
- A double strand break is made at the genomic location of interest
- The cell repair of the DSB allows
  - Small insertions and deletions form, in a coding region this can cause frameshift mutations
  - Homology directed repair incorporates a donor template sequence





Genome Engineering with CRISPR-Cas9

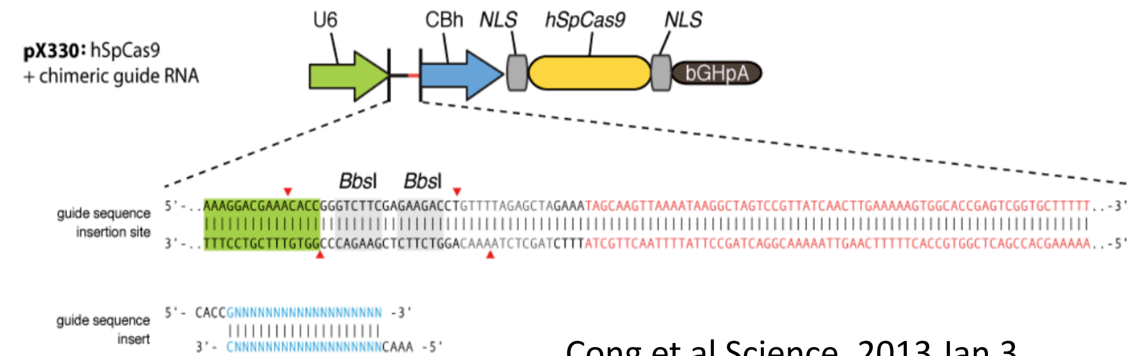
- guideRNA
  - 20 nucleotide recognition site next to a PAM (NGG)
  - Scaffold that interacts with Cas9
- Cas9 protein
  - Generates DSB 3bp upstream from the PAM site



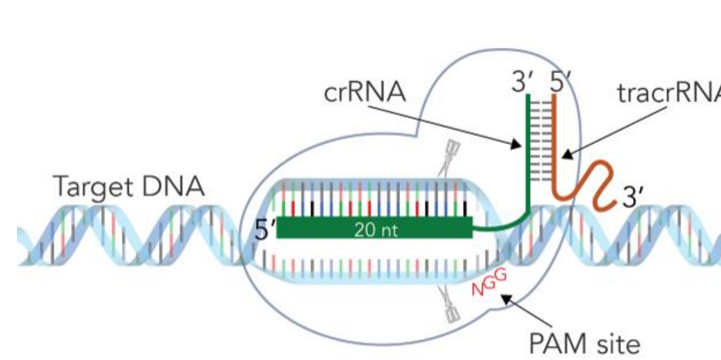
Mark Miller

guideRNA and Cas9 formats

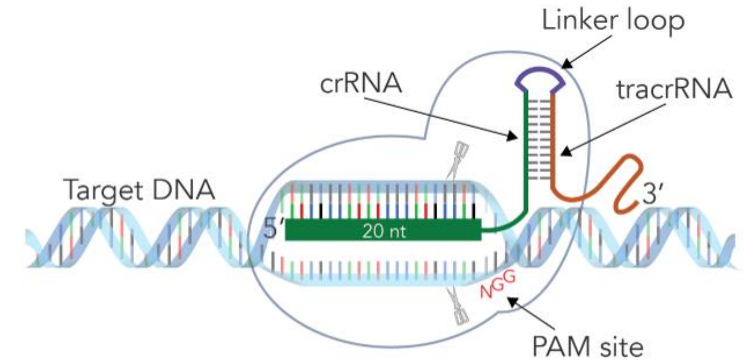
- Plasmid expressing guideRNA and Cas9
  - Single guideRNA with promoter
  - Cas9 with promoter
- Ribonucleoprotein (RNP)
  - crRNA + tracrRNA annealed to form sgRNA
  - Synthetic single guideRNA
  - Cas9 Protein



Cong et al Science. 2013 Jan 3.



A. 2-part crRNA:tracrRNA complex

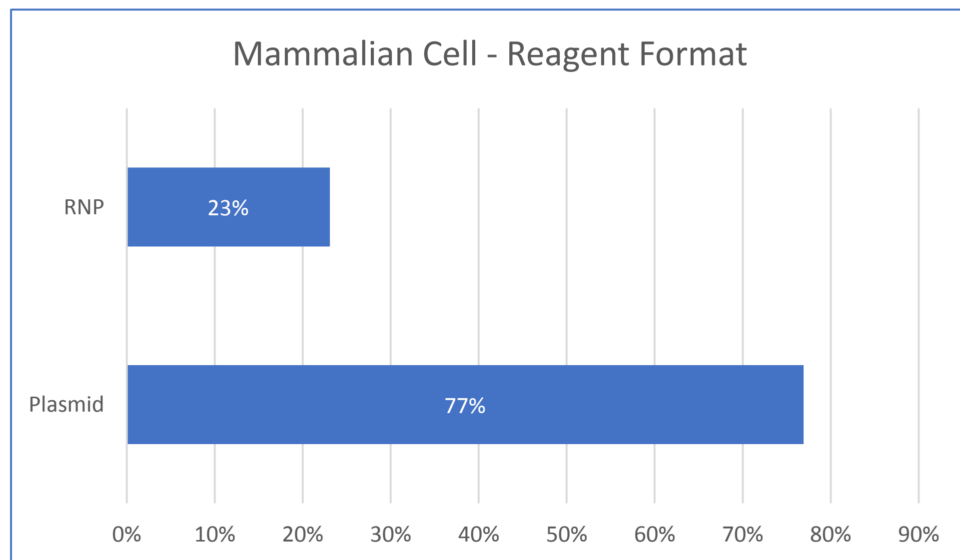


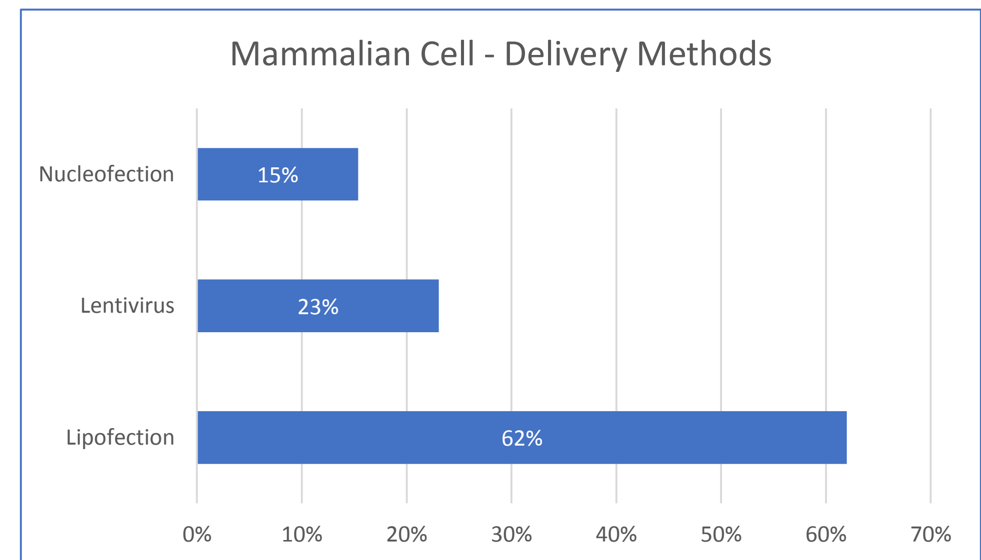
B. Single fusion sgRNA trigger

IDT
INTEGRATED DNA TECHNOLOGIES

GERG 2017 Study

- CRISPR/Cas9 Methods: Preferences from the Field
- Plasmid with Lipofection was the most popular combination for mammalian cell work





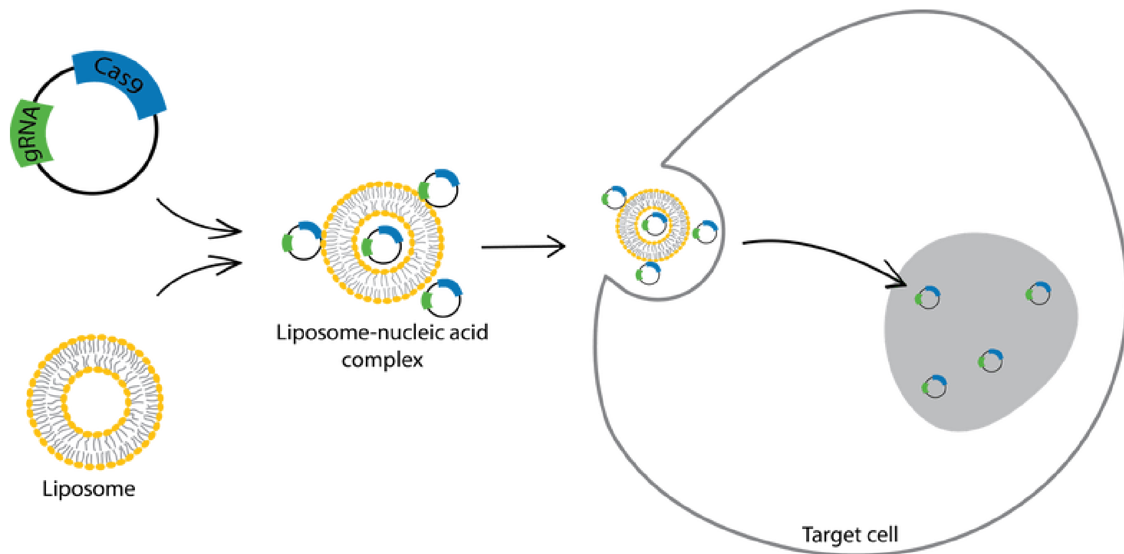
https://abrf.org/research-group/genome-editing-research-group-gerg

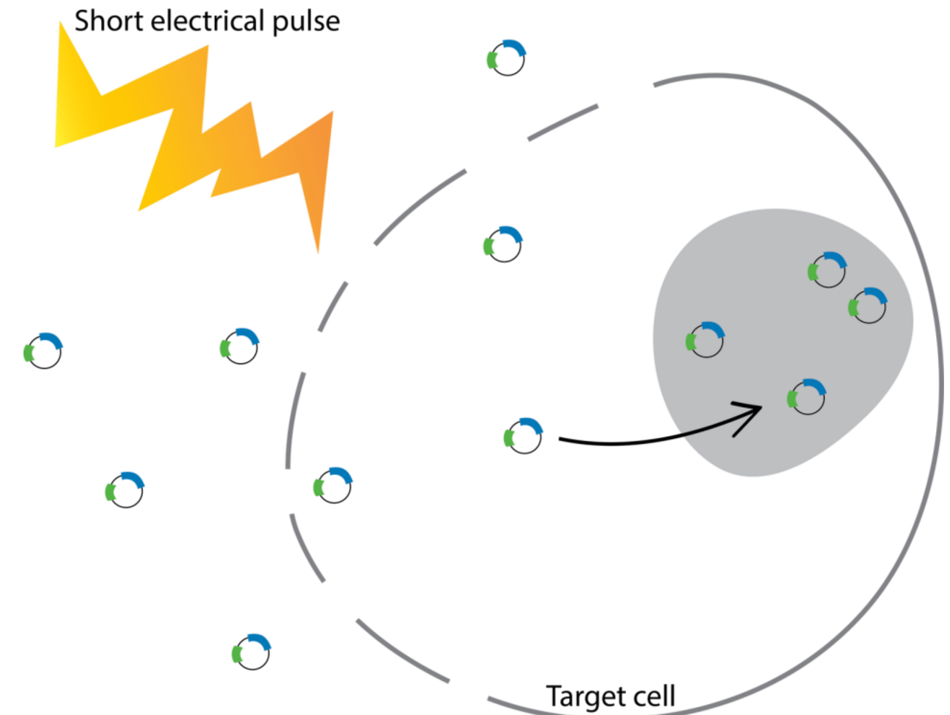
Plasmid vs RNP

- Cells that are amenable to transfection or viral transduction
- Optimal promoters for Cas9 and guideRNA must be cloned into plasmid
- Cas9 must be transcribed and translated from plasmid and takes longer to act
- Cas9 expression persists longer from a plasmid
- Plasmid DNA can become randomly integrated in the genome

- Use of nucleofection can deliver to many cell types, including primary cells
- Cas9 protein is organism independent, helpful to cores with multiple organisms
- Cas9 protein is ready to act at delivery
- RNP is degraded after 24 hours
- Cannot integrate in the genome

Lipofection vs Nucleofection

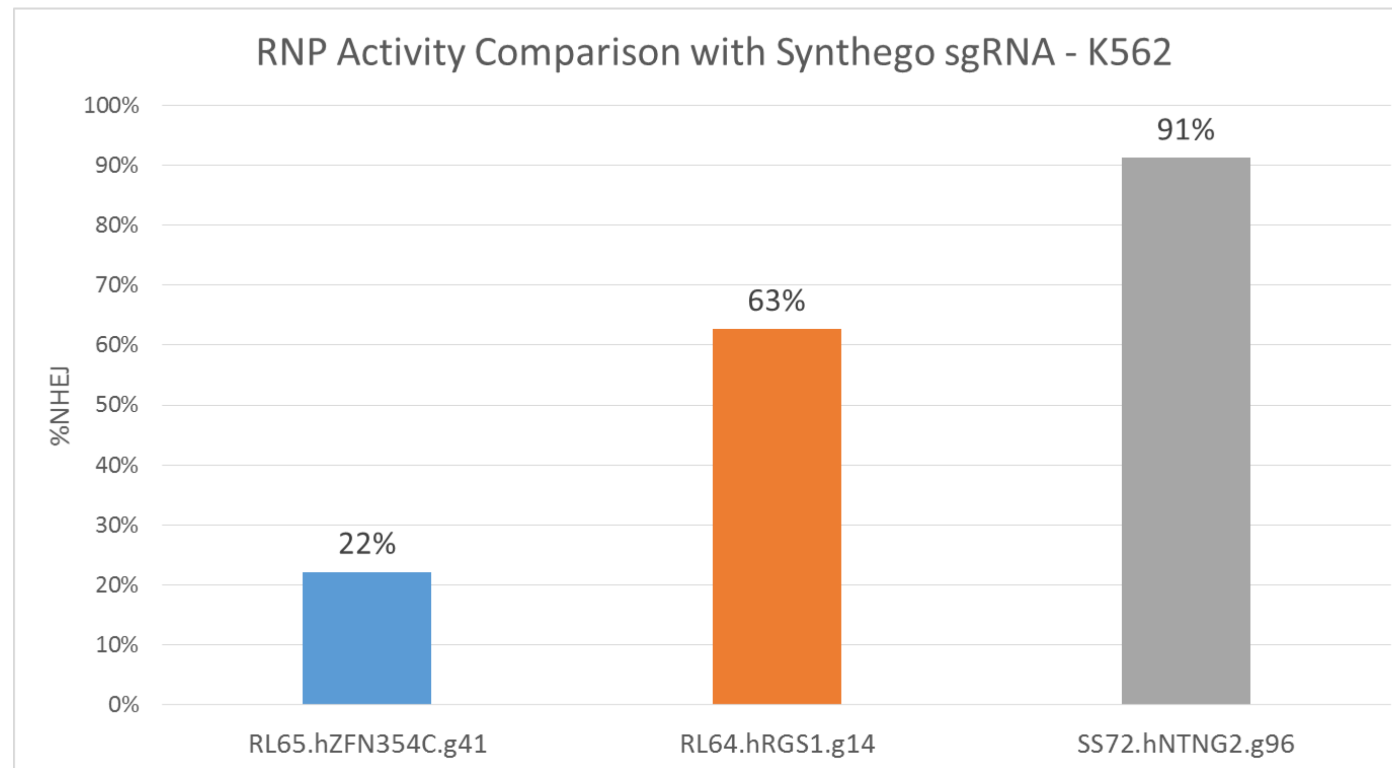




https://blog.benchling.com/2016/03/24/how-to-express-crispr-in-your-target-cells/

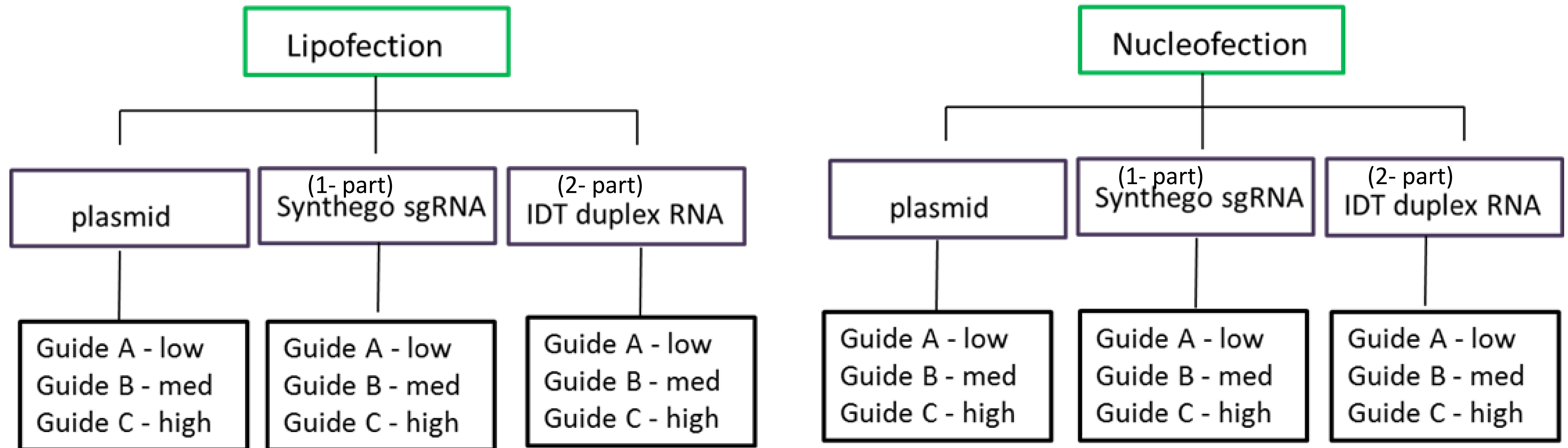
RNP Activity Comparison with Synthego sgRNA Low-Medium-High

Cell Line	sgRNA Type	RNP Ratio (sgRNA:Cas9)	Cas9 Protein Amount (pmol)	# Cells/nucleofection	Lonza 4D Nucleofector Program	Cuvette	Solution
K562	Modified	3:1	25pmol	50,000	FF-120	Small	P3



Shondra Miller

GERG Study Proposal

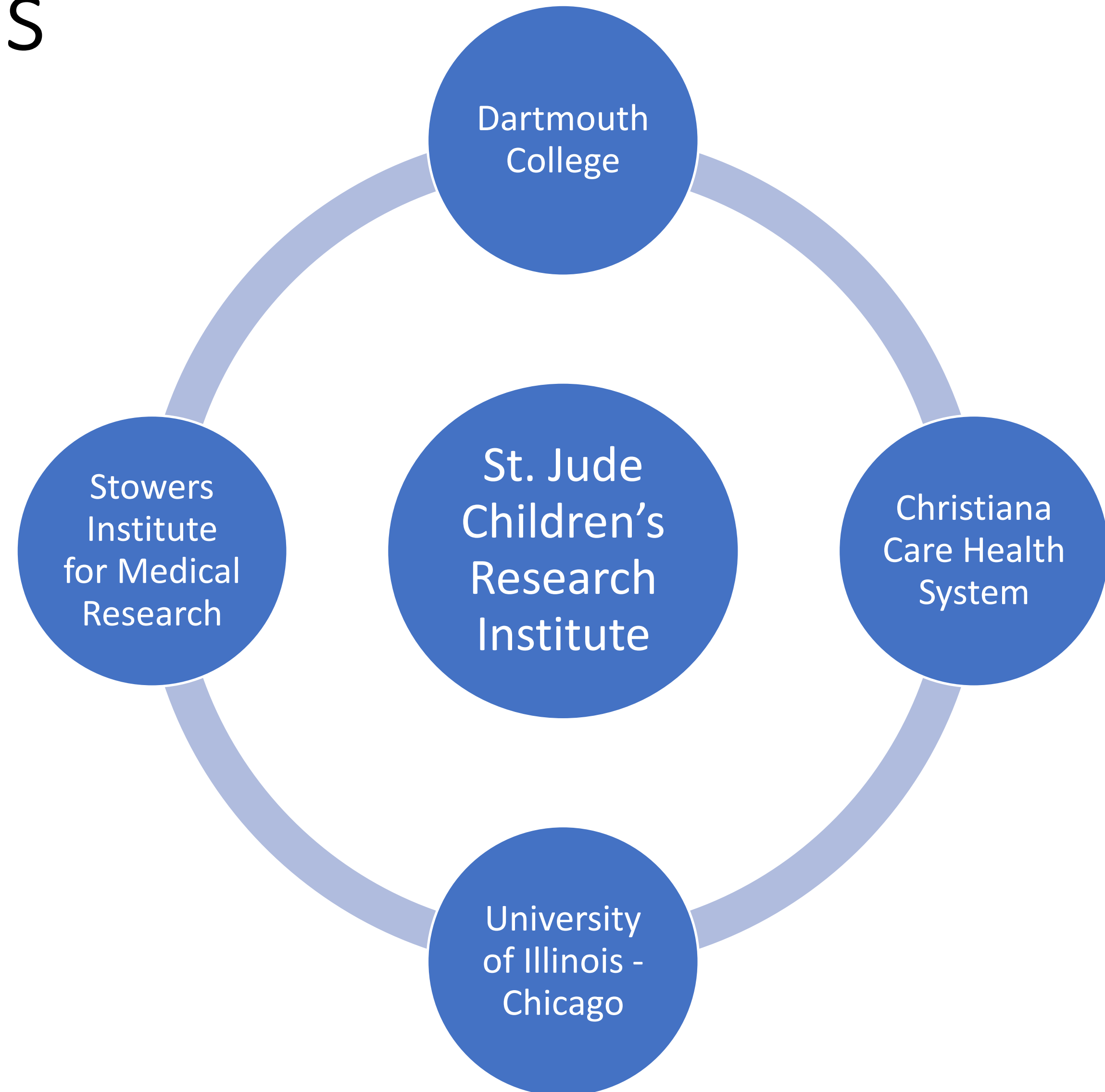


All experiments were done on HEK293 cells
LipoD293 transfection reagent for lipofections
Lonza-IIB or Lonza-4D nucleofector
Cas9 protein from Synthego
All samples were collected and sent out for Next-generation sequencing (NGS)

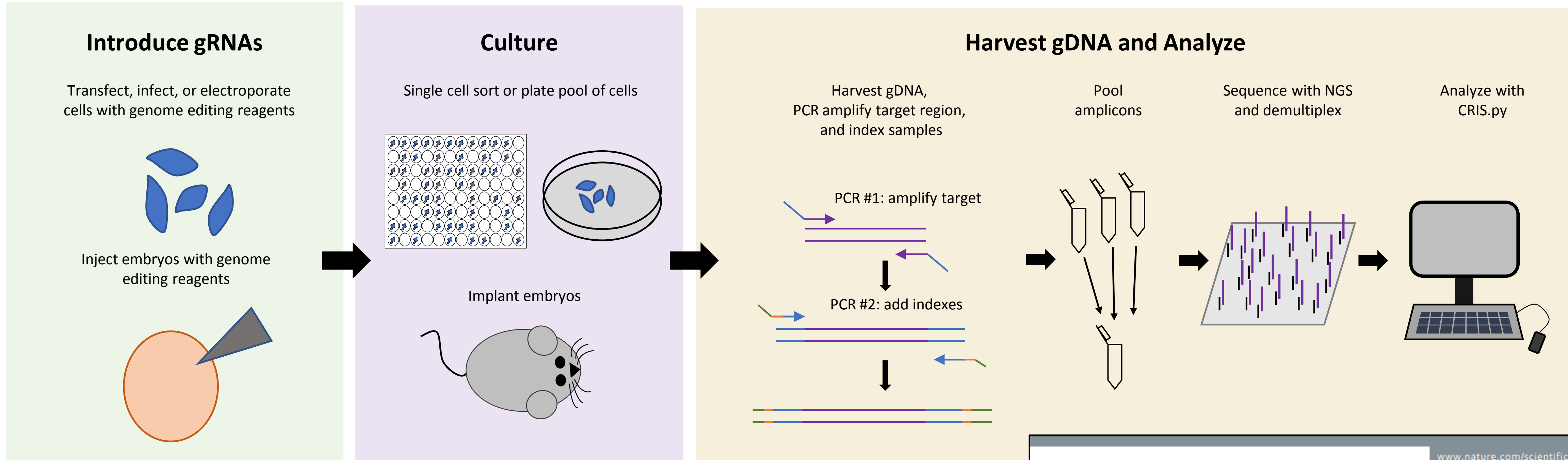
Shirin Modarai

GERG Lab Participants

- 3 gRNA that were previously identified as low, medium, high activity
- PX330 plasmids cloned
  - Expresses guideRNA and Cas9
- Donations and discounts
  - 2-part gRNA
  - sgRNA
  - Cas9 protein
- Cell line and reagents sent to participants
- 4 sites performed cell experiments
- 1 site performed targeted amplicon NGS for indel analysis



Genome editing workflow with NGS analysis



www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

CRIS.py: A Versatile and High-throughput Analysis Program for CRISPR-based Genome Editing

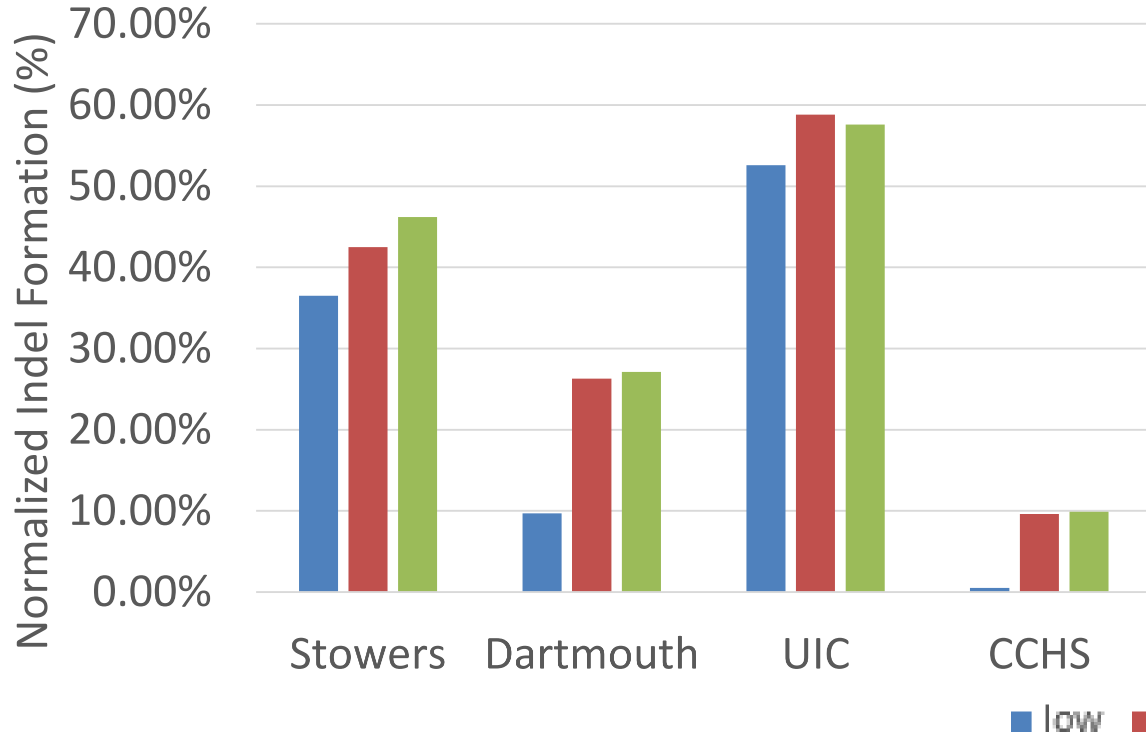
Received: 28 November 2018
Accepted: 25 February 2019
Published online: 12 March 2019

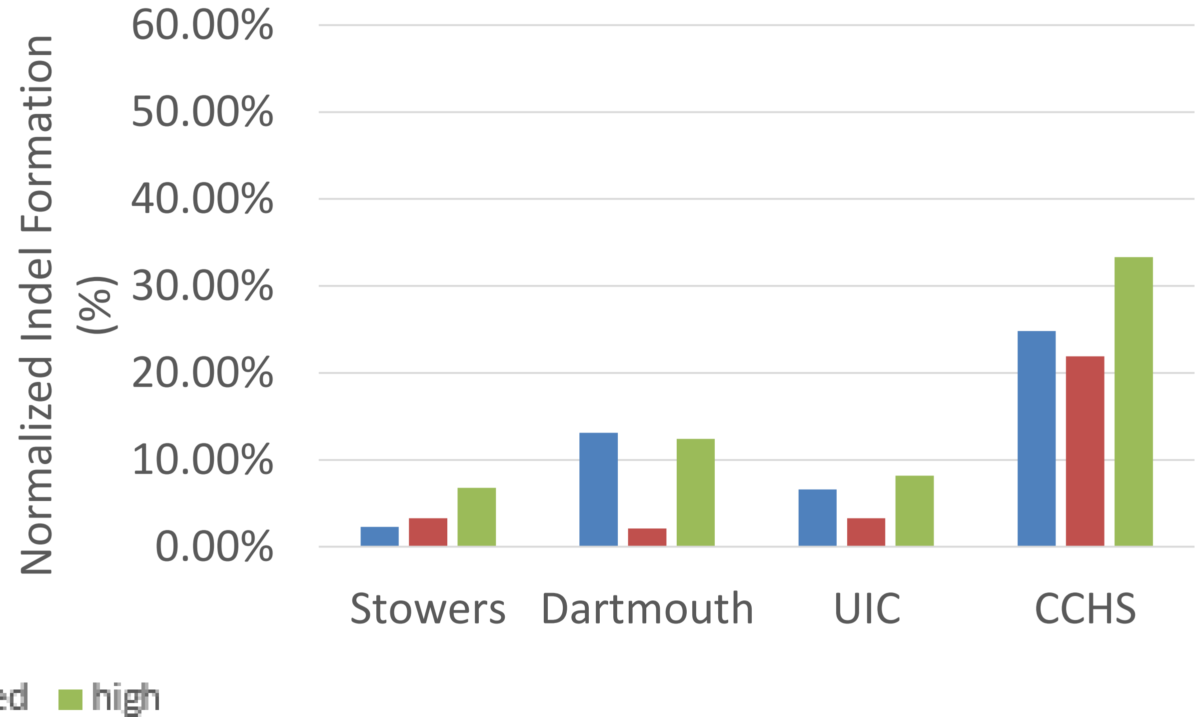
Jon P. Connelly[1,2] & Shondra M. Pruett-Miller[1,2]

CRISPR-Cas9 technology allows the creation of user-defined genomic modifications in cells and whole organisms. However, quantifying editing rates in pools of cells or identifying correctly edited clones

Shondra Miller

Plasmid





low med high

	Rxn volume	plasmid [ug]	Cells/rxn
Stowers	----	1	500,000
Dartmouth	----	1	500,000
UIC	----	1	500,000
CCHS	----	1	500,000

	Rxn volume	plasmid [ug]	Cells/rxn
Stowers	100ul	2	1,000,000
Dartmouth	100ul	2	1,000,000
UIC	100ul	2	1,000,000
CCHS	20ul	0.5	250,000

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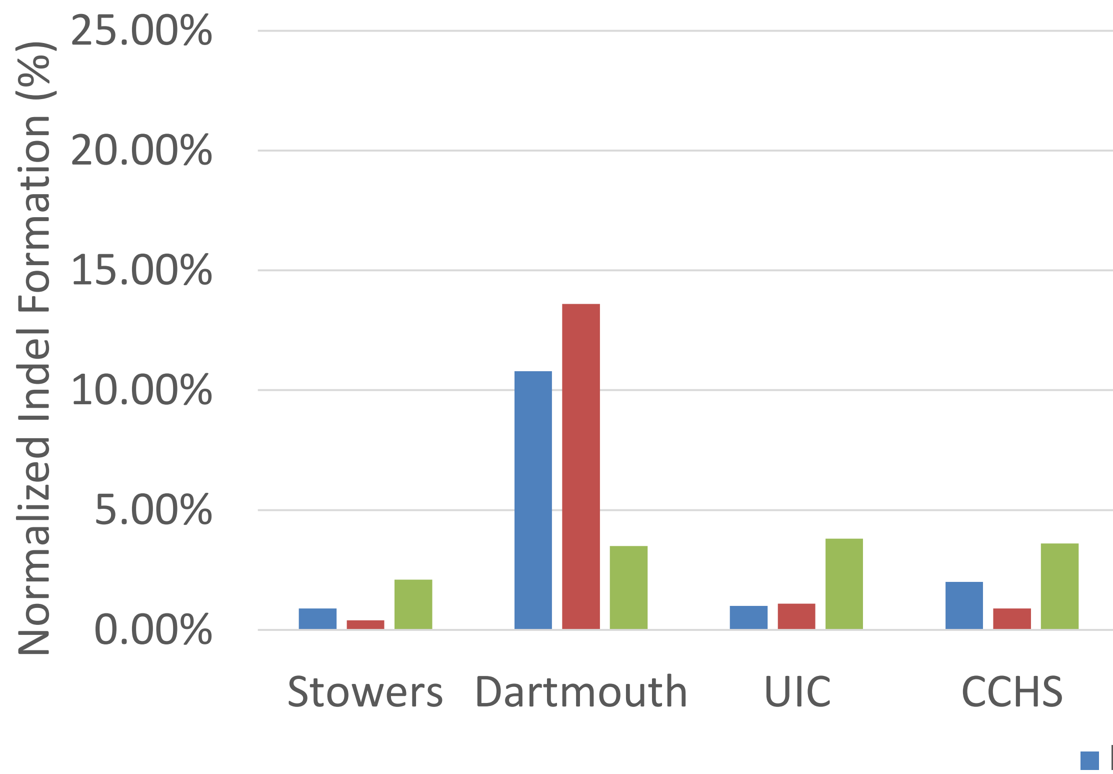
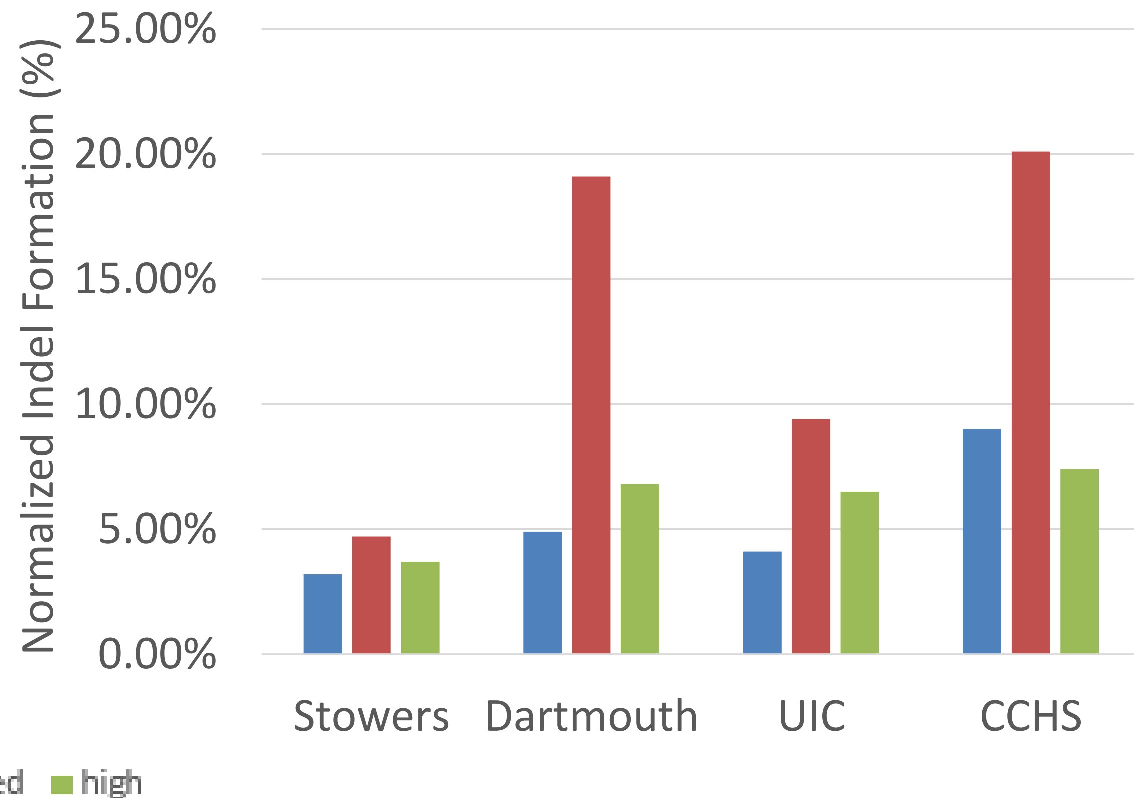
2-Part gRNA





■ low ■ med ■ high

	Rxn volume	Guide RNA:Cas9 Protein [pmol]	Ration of RNA:protein	Cells/rxn
Stowers	----	24:24	1	500,000
Dartmouth	----	6:6	1	160,000
UIC	----	6:6	1	160,000
CCHS	----	6:6	1	160,000

	Rxn volume	Guide RNA: Cas9 protein [pmol]	Ratio of RNA:protein	Cells/rxn
Stowers	100ul	160:139	1	460,000
Dartmouth	100ul	480:416	1	1,400,000
UIC	100ul	160:139	1	460,000
CCHS	20ul	120:104	1	350,000

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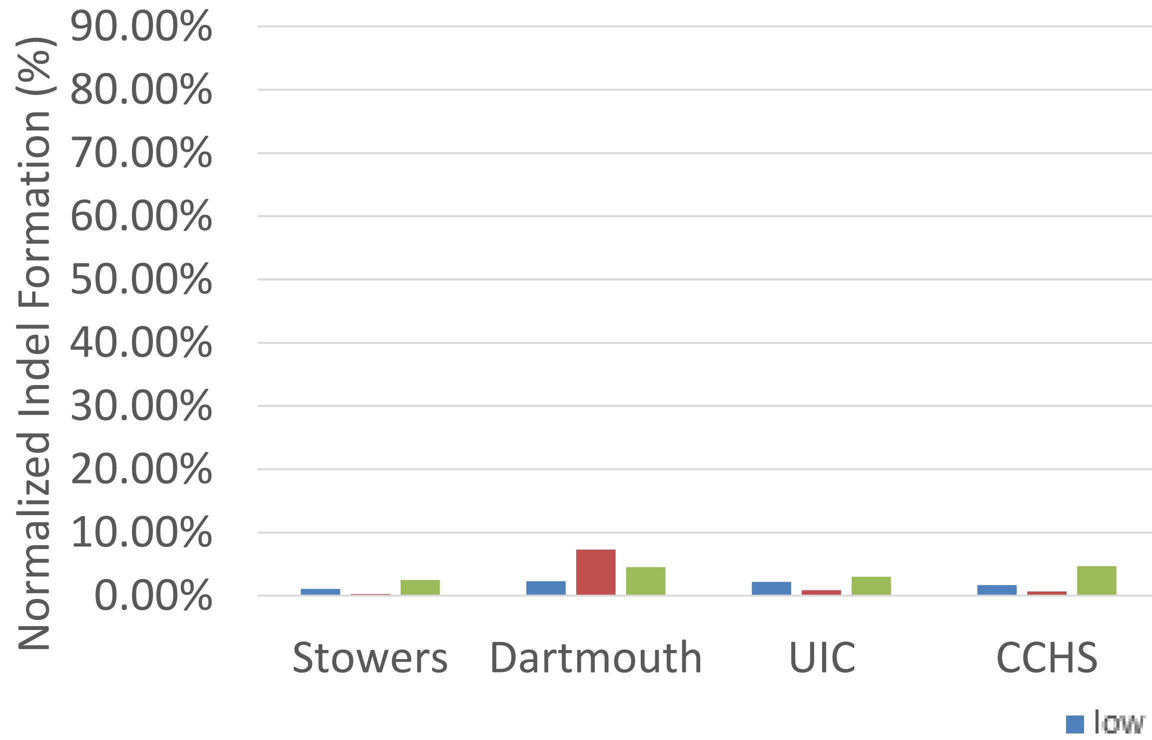
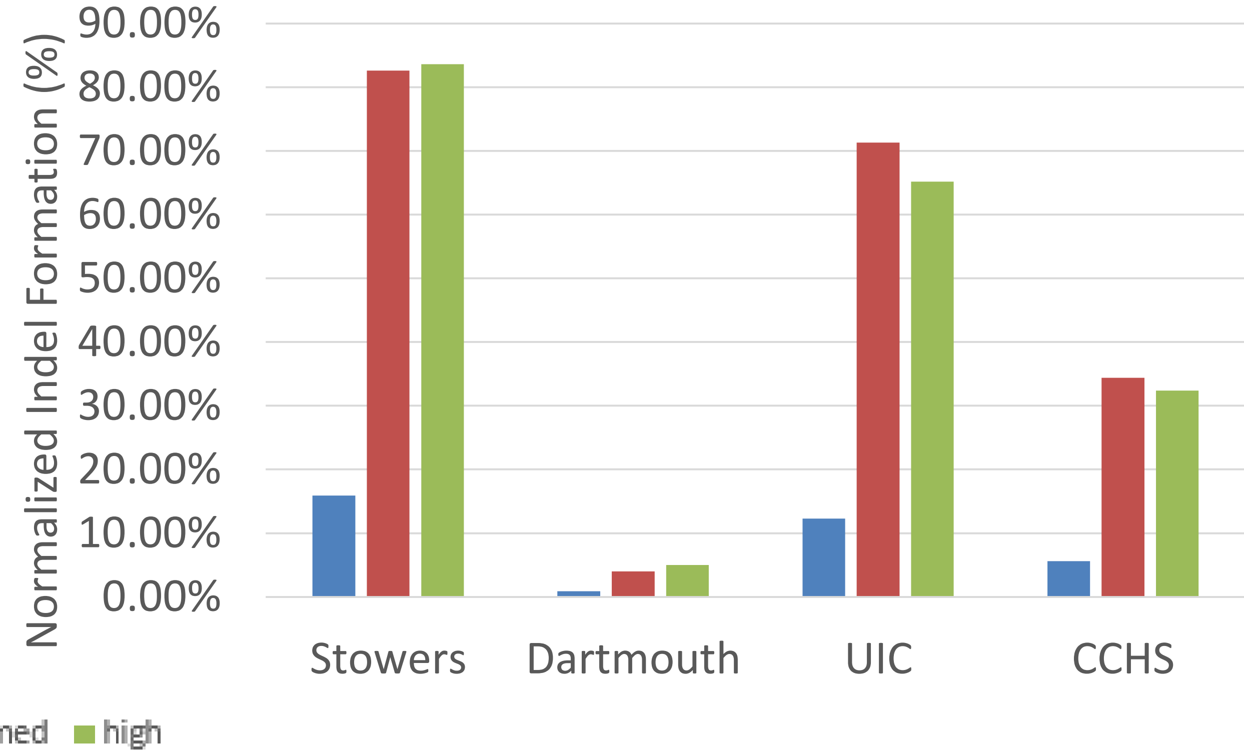
1-Part sgRNA





	Rxn volume	Guide RNA: Cas9 protein [pmol]	Ratio of RNA:protein	Cells/rxn
Stowers	----	15.6:12	1.3:1	500,000
Dartmouth	----	3.9:3	1.3:1	120,000
UIC	----	3.9:3	1.3:1	120,000
CCHS	----	3.9:3	1.3:1	120,000

	Rxn volume	Guide RNA: Cas9 protein [pmol]	Ratio of RNA:protein	Cells/rxn
Stowers	100ul	720:80	9:1	600,000
Dartmouth	100ul	720:80	9:1	600,000
UIC	100ul	720:80	9:1	600,000
CCHS	20ul	180:20:00	9:1	200,000

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Most Reproducible: Nucleofection + 1-part sgRNA

	Stowers	Dartmouth	UIC	CCHS
Delivery method with highest indel rate	nucleofection	lipofection	nucleofection	nucleofection
GuideRNA format with highest indel rate	sgRNA	plasmid	sgRNA	sgRNA

Experience Level

Delivery Method + Reagent Format	Stowers Experience	Stowers Highest Indel Rate	Dartmouth Experience	Dartmouth Highest Indel Rate	UIC Experience	UIC Highest Indel Rate	CCHS Experience	CCHS Highest Indel Rate
Lipofection + Plasmid	beginner (1-10 transfections)	46.2	**master (50+ transfections)**	**27.1**	intermediate (10-50 transfections)	58.8	intermediate (10-50 transfections)	9.9
Lipofection + 2 part gRNA	beginner (1-10 transfections)	2.1	beginner (1-10 transfections)	13.6	intermediate (10-50 transfections)	3.8	beginner (1-10 transfections)	3.6
Lipofection + 1 part sgRNA	beginner (1-10 transfections)	2.5	beginner (1-10 transfections)	7.3	beginner (1-10 transfections)	3	beginner (1-10 transfections)	4.7
Nucleofection + Plasmid	beginner (1-10 transfections)	6.8	beginner (1-10 transfections)	13.1	beginner (1-10 transfections)	8.2	master (50+ transfections)	33.3
Nucleofection + 2 part gRNA	beginner (1-10 transfections)	4.7	beginner (1-10 transfections)	19.1	beginner (1-10 transfections)	9.4	master (50+ transfections)	20.1
Nucleofection + 1 part sgRNA	**beginner (1-10 transfections)**	**83.6**	beginner (1-10 transfections)	5	**beginner (1-10 transfections)**	**71.3**	**master (50+ transfections)**	**34.4**

GERG Study Conclusions

- Lipofection worked best with plasmid
- Nucleofection worked best with RNP
- Nucleofection + sgRNA had highest indel rates overall
  - Was the most reproducible
  - Worked well for beginners
- Indel rates varied across all methods, all sites
  - If your guideRNA results aren't ideal, try another method
- Standard Operating Procedures are needed for RNP delivery
  - Difficult to determine the preferred amount to use for each method
  - Even after discussion, we still did not use exact same values
  - Adjustments based on reaction volumes, cells, equipment available

Acknowledgements

Elizabeth Sergison – Dartmouth

Brandon Miller – Stowers

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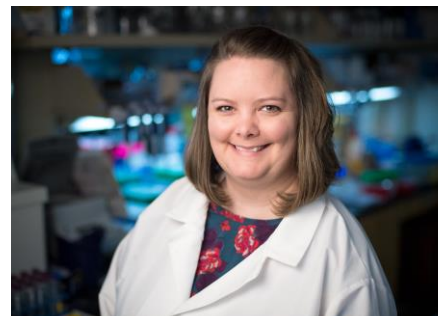
Maureen Reagan – UIC

Shondra Miller – St. Jude

Shaina Porter – St. Jude



***Scientific Session Monday, 1:00-2:30 - CRISPR/CAS TECHNOLOGY**

****Poster 154 – Monday, 2:30 - 3:30 p.m.**