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
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


A. 2-part crRNA:tracrRNA complex
B. Single fusion sgRNA trigger
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
RNP Activity Comparison with Synthego sgRNA Low-Medium-High

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

RNP Activity Comparison with Synthego sgRNA - K562

- RL65.hZFN354C.g41: 22%
- RL64.hRG51.g14: 63%
- SS72.hNTNG2.g96: 91%

Shondra Miller
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)
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

**Introduce gRNAs**
Transfect, infect, or electroporate cells with genome editing reagents

**Inject embryos with genome editing reagents**

**Culture**
Single cell sort or plate pool of cells

**Implant embryos**

**Harvest gDNA and Analyze**
Harvest gDNA, PCR amplify target region, and index samples

**Pool amplicons**

**Sequence with NGS and demultiplex**

**Analyze with CRIS.py**

Shondra Miller
Plasmid

<table>
<thead>
<tr>
<th>Institution</th>
<th>Rxn Volume</th>
<th>Plasmid [ug]</th>
<th>Cells/rxn</th>
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</thead>
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<tr>
<td>Stowers</td>
<td>100ul</td>
<td>2</td>
<td>1,000,000</td>
</tr>
<tr>
<td>Dartmouth</td>
<td>100ul</td>
<td>2</td>
<td>1,000,000</td>
</tr>
<tr>
<td>UIC</td>
<td>100ul</td>
<td>2</td>
<td>1,000,000</td>
</tr>
<tr>
<td>CCHS</td>
<td>20ul</td>
<td>0.5</td>
<td>250,000</td>
</tr>
</tbody>
</table>

Lipofection

Nucleofection

Normalized Indel Formation (%)

Stowers | Dartmouth | UIC | CCHS

Normalized Indel Formation (%)

Stowers | Dartmouth | UIC | CCHS

Shirin Modarai, Elizabeth Sergison
2-Part gRNA

Lipofection

<table>
<thead>
<tr>
<th></th>
<th>Stowers</th>
<th>Dartmouth</th>
<th>UIC</th>
<th>CCHS</th>
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</thead>
<tbody>
<tr>
<td>Rxn volume</td>
<td>100ul</td>
<td>100ul</td>
<td>100ul</td>
<td>20ul</td>
</tr>
<tr>
<td>Ratio of RNA:protein</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cells/rxn</td>
<td>500,000</td>
<td>160,000</td>
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</table>

Nucleofection

<table>
<thead>
<tr>
<th></th>
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<th>Dartmouth</th>
<th>UIC</th>
<th>CCHS</th>
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<td>Rxn volume</td>
<td>100ul</td>
<td>100ul</td>
<td>100ul</td>
<td>20ul</td>
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<tr>
<td>Ratio of RNA:protein</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Cells/rxn</td>
<td>460,000</td>
<td>1,400,000</td>
<td>460,000</td>
<td>350,000</td>
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</table>

Shirin Modarai, Elizabeth Sergison
1-Part sgRNA

**Lipofection**

- Stowers: 0.00%
- Dartmouth: 10.00%
- UIC: 20.00%
- CCHS: 30.00%

**Nucleofection**

- Stowers: 0.00%
- Dartmouth: 10.00%
- UIC: 20.00%
- CCHS: 30.00%

**Table**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Stowers</td>
<td>100ul</td>
<td>720:80</td>
<td>9:1</td>
<td>600,000</td>
</tr>
<tr>
<td>Dartmouth</td>
<td>100ul</td>
<td>720:80</td>
<td>9:1</td>
<td>600,000</td>
</tr>
<tr>
<td>UIC</td>
<td>100ul</td>
<td>720:80</td>
<td>9:1</td>
<td>600,000</td>
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<tr>
<td>CCHS</td>
<td>20ul</td>
<td>180:20:00</td>
<td>9:1</td>
<td>200,000</td>
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</tbody>
</table>

Shirin Modarai, Elizabeth Sergison
Most Reproducible: Nucleofection + 1-part sgRNA

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<thead>
<tr>
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<th>Dartmouth</th>
<th>UIC</th>
<th>CCHS</th>
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</thead>
<tbody>
<tr>
<td>Delivery method with highest indel</td>
<td>nucleofection</td>
<td>lipofection</td>
<td>nucleofection</td>
<td>nucleofection</td>
</tr>
<tr>
<td>rate</td>
<td></td>
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<tr>
<td>GuideRNA format with highest indel</td>
<td>sgRNA</td>
<td>plasmid</td>
<td>sgRNA</td>
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<tr>
<td>rate</td>
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<tr>
<td>Delivery Method + Reagent Format</td>
<td>Stowers Experience</td>
<td>Stowers Highest Indel Rate</td>
<td>Dartmouth Experience</td>
<td>Dartmouth Highest Indel Rate</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------------</td>
<td>---------------------------</td>
<td>----------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Lipofection + Plasmid</td>
<td>beginner (1-10 transfections)</td>
<td>46.2</td>
<td>master (50+ transfections)</td>
<td>27.1</td>
</tr>
<tr>
<td>Lipofection + 2 part gRNA</td>
<td>beginner (1-10 transfections)</td>
<td>2.1</td>
<td>beginner (1-10 transfections)</td>
<td>13.6</td>
</tr>
<tr>
<td>Lipofection + 1 part sgRNA</td>
<td>beginner (1-10 transfections)</td>
<td>2.5</td>
<td>beginner (1-10 transfections)</td>
<td>7.3</td>
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<tr>
<td>Nucleofection + Plasmid</td>
<td>beginner (1-10 transfections)</td>
<td>6.8</td>
<td>beginner (1-10 transfections)</td>
<td>13.1</td>
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<tr>
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<td>4.7</td>
<td>beginner (1-10 transfections)</td>
<td>19.1</td>
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<tr>
<td>Nucleofection + 1 part sgRNA</td>
<td><strong>beginner (1-10 transfections)</strong></td>
<td><strong>83.6</strong></td>
<td>beginner (1-10 transfections)</td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>
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
Shirin Modarai – CCHS
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.