Size limitation for electroporation of DNA constructs in mouse embryos

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Abstract

The use of CRISPR/Cas9 genome editing has led to major advancements in the generation of genetically engineered animal models. CRISPR/Cas9 reagents are commonly delivered by microinjection (MI) directly into embryos, however, MI is technically demanding, expensive and time consuming. Electroporation (EP) offers a cost effective, simple, and efficient alternative to MI. The objective of this study is to evaluate the success of electroporating DNA constructs of varying sizes into embryos. We hypothesize that small DNA constructs ≤ 200 bp will be electroporated efficiently while larger DNA constructs (≥500 bp) will undergo EP less efficiently with increasing size. One-cell embryos will be collected from superovulated, immature C57BL/6 mice, then assigned to 6 experimental groups. Four groups will undergo EP with varying sizes of DNA constructs (200, 500, 700, and 1100 bp) + CRISPR/Cas9 reagents. Control groups will include non-electroporated embryos and a group that will be electroporated without DNA constructs. Embryos will be cultured *in vitro*, monitored for continued survival and development and collected as blastocysts for PCR analysis to assess genomic integration of the DNA constructs. Statistical analysis will be done using a one-way ANOVA with a Tukey post-hoc test. We expect to see a negative correlation between the number of embryos carrying a genomic integration with the increasing size of the electroporated DNA construct. This would suggest that there may be size limitations for EP and provide guidelines for when alternative methods of delivery of larger DNA constructs should be considered.

Objective and Hypothesis

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- We hypothesize that small DNA constructs ≤ 200 bp will be electroporated efficiently while larger DNA constructs (≥500 bp) will undergo EP less efficiently with increasing size.

Methods

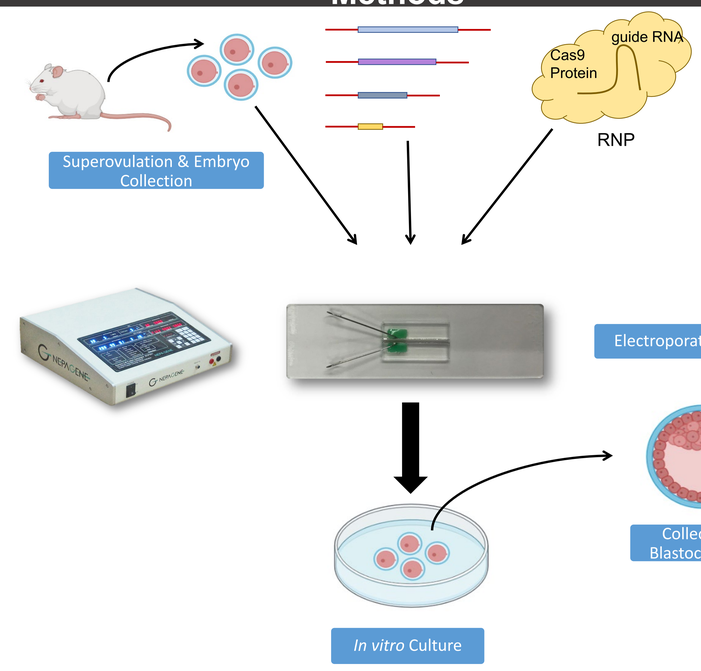


Figure 1. Immature (3-4 weeks of age) female FVB/NJ mice were superovulated with intraperitoneal (IP) injections of 5 IU PMSG followed 48 hours later by IP injections of 5 IU hCG/overnight mating. Embryos were collected 12 hours after hCG/mating. Donor DNA constructs of either 200, 500, 700 or 1100 bp and Cas9/sgRNA ribonucleoprotein complexes (RNPs) are added to Opti-MEM to create the electroporation reagent. Embryos were cultured *in vitro* to blastocysts then collected for DNA extraction followed by PCR analysis.

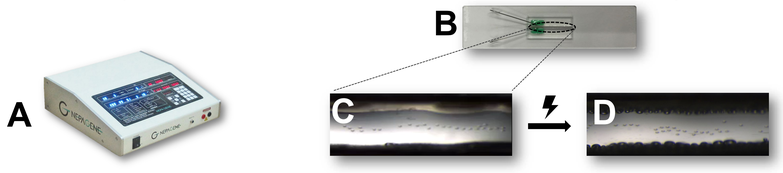


Figure 2. Electroporation of embryos. **A.** The NepaGene NEPA21 Type II electroporator. **B.** 1mm gap glass slide electrode. **C.** Embryos are loaded between the electrodes with 5 µL of electroporation reagent. **D.** Electrical pulses cause intracellular movement of DNA constructs and Cas9/sgRNA RNP complexes. The formation of bubbles on the electrodes indicates electroporation is occurring.

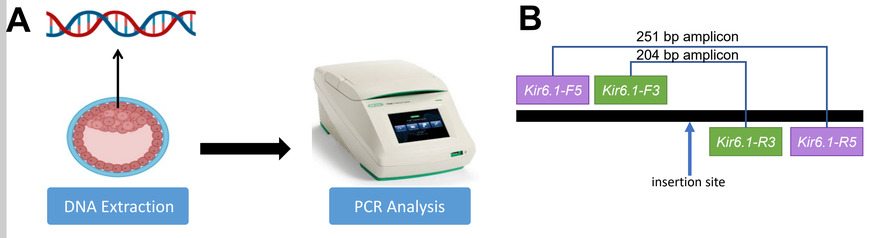


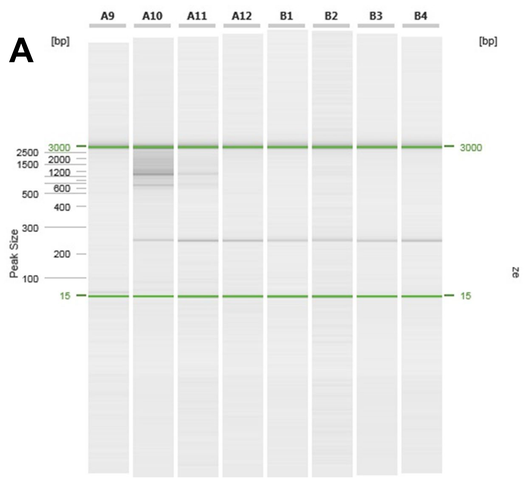
Figure 3. PCR analysis to determine electroporation efficiency. **A.** Blastocysts were collected in 10 µL of water and DNA was extracted using a crude lysis preparation. PCR analysis was done to assess successful electroporation of each donor DNA construct. **B.** PCR primers *Kir6.1-F5* and *Kir6.1-R5* have a 251 bp expected amplicon in wild type blastocysts. PCR primers *Kir6.1-F3* and *Kir6.1-R3* have a 204 bp expected amplicon in wild type blastocysts.

Results

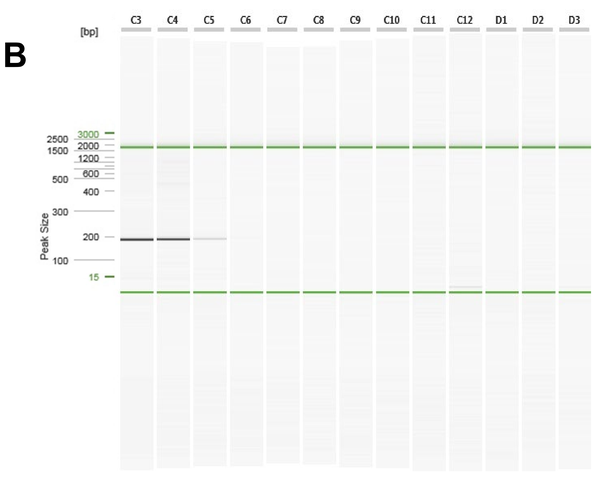
Table 1. Embryo survival and developmental rates after electroporation with donor DNA constructs.

Group	Zygotes	2-cell (%)	4-cell (%)	8-cell (%)	Morula (%)	Blastocyst (%)
Control (untreated)	28	20 (71)	19 (68)	19 (68)	19 (68)	19 (68)
Electroporation Control	27	16 (59)	13 (48)	13 (48)	12 (44)	10 (37)
200 bp Construct	49	31 (63)	25 (51)	21 (43)	21 (43)	21 (43)
1100 bp Construct	49	32 (65)	28 (57)	28 (57)	27 (55)	26 (53)

Percentage at each stage was calculated as the number of embryos at that stage/initial number of zygotes for that experimental group.



Lane	°Celsius
A9	50.0
A10	51.4
A11	53.8
A12	57.5
B1	62.0
B2	65.9
B3	68.5
B4	70.0



Lane	Genomic Copies
C3	1.4×10^{13}
C4	1.4×10^{12}
C5	1.4×10^{11}
C6	1.4×10^{10}
C7	1.4×10^{9}
C8	1.4×10^{8}
C9	1.4×10^{7}
C10	1.4×10^{6}
C11	140,000
C12	14,000
D1	1,400
D2	140
D3	14

Figure 4. Primer optimization for genotyping assay. **A.** Primer annealing temperature gradient assay for *Kir6.1-F5* and *Kir6.1-R5.* Lanes A9-B4 contained wild type DNA extracted from control mouse embryos. PCR amplification was done on identical replicate samples using annealing temperatures ranging from 50-70 ºC. Amplification was successful at the majority of annealing temperatures tested. **B.** Primer sensitivity assay for *Kir6.1-F5* and *Kir6.1-R5.* Lanes C4-D3 contained PCR reactions with varying copy numbers of genomic DNA to test sensitivity of the assay. PCR using primers *Kir6.1-F5* and *Kir6.1-R5* had successful amplification for samples containing ≥ 1.4×10^{11} copies of the target sequence. Since blastocysts are expected to contain many fewer target DNA sequences (~200 copies) this result guided our decision to develop a nested PCR amplification strategy.

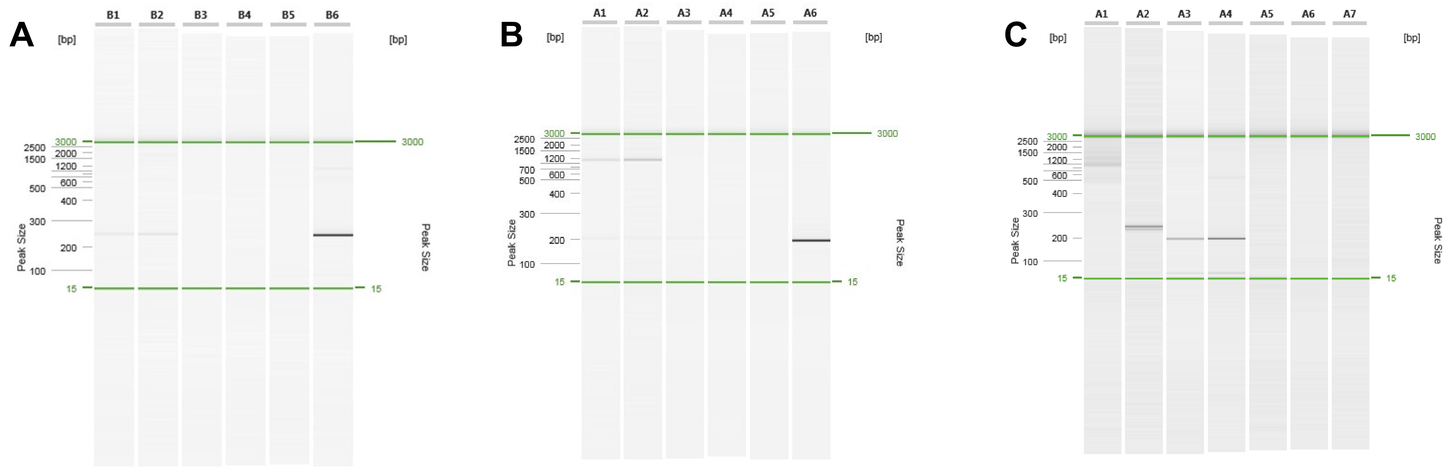


Figure 5. Genotyping assay used to detect the knock-in of donor DNA constructs. **A.** Assay validation for primers *Kir6.1-F5* and *Kir6.1-R5*. Lanes B1-4 are untreated wild type control embryos showing variable amplification, which is not unexpected due to the sensitivity of the assay. Lane B5 is a negative control (no DNA template) and showed no amplification. Lane B6 is positive control using ~40 ng of C57BL/6 genomic DNA, showed successful amplification of the expected 251 bp wild type PCR amplicon. **B**. Assay validation for primers *Kir6.1-F3* and *Kir6.1-R3*. Lanes A1-4 contain untreated wild type control embryos, again showing variable amplification. Lanes A5 is a negative (no DNA template). Lane A6 is a positive control (~40 ng of C57BL/6 genomic DNA), which showed successful amplification of the expected 204 bp amplicon. **C.** Nested PCR assay to further increase amplification of target allele. PCR amplification was done using *Kir6.1-F5* and *Kir6.1-R5* primers. This reaction was then used as a template for nested PCR using *Kir6.1-F3* and *Kir6.1-R3* primers to maximize the number of amplicons of interest for each wild type mouse blastocyst. Lanes A1-4 showed inconsistent amplification. Lanes A5-6 contain negative controls (no DNA template) and showed no amplification. Lane A7 was the positive control (template from Panel A, Lane B6) and was expected to show the nested 204 bp amplicon. It did not amplify.

Expected Conclusions & Future Directions

- Once assay validation and optimization are complete, we expect to have a sensitive and specific nested PCR assay that can reliably detect both the wild type and the knock in alleles from mouse blastocysts generated in these experiments.
- We expect to detect evidence of successful genomic integration of the 200 bp construct based on PCR analysis of electroporated embryos but see fewer embryos with successful integration of the 1100 bp construct. This would indicate less efficient electroporation and/or less efficient genomic integration of the larger construct.
- If we do see a difference in integration of the 200 versus 1100 bp constructs, we will repeat the experiments using 500-700 bp constructs to assess the efficiency of electroporation/integration of intermediate size constructs.

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