CRISPR editing validation, immunostaining and DNA sequencing of individual fixed bovine embryos

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CRISPR technologies used for mammalian embryology have wide implications from basic research to applications in agriculture and biomedicine. Confirmation of successful gene editing following CRISPR/Cas9 delivery is often limited to either protein expression or sequencing analyses of embryos but not both, due to technical challenges. Herein we report an integrative approach for evaluating both protein expression and genotype of single embryos from fixed bovine embryos previously subjected to CRISPR/Cas9 microinjection. The techniques described facilitate investigation of functional genomics in bovine embryos compatible with gene editing in livestock after zygotic CRISPR microinjection. These methods avoid traditional avenues that necessitate the use of gene-edited cell lines followed by nuclear transfer that hinder efficiency, limit physiological relevance and contribute to technical challenges.

Confirmation of genome editing is often required after microinjection of CRISPR/Cas9 components into mammalian zygotes [1–4]. However, functional studies of proteins during embryogenesis associated with gene editing often require embryo fixation [5], thereby hindering the recovery of high-quality DNA suitable for DNA extraction and sequencing for genotype confirmation. We previously established a method for sequencing individual mammalian embryos following CRISPR/Cas9 zygotic microinjection [6]. Expanding upon this technique, our objective was to (i) develop a method to evaluate and recover fixed bovine blastocysts subjected to immunohistochemistry and (ii) extract DNA from fixed and imaged embryos suitable for DNA sequencing. Collectively, our methods represent a significant advancement towards integrating genome-editing strategies into the rapidly expanding field of livestock research [7].

Unless otherwise specified, reagents were purchased from Sigma-Aldrich, MI, USA. All washes and incubations are

performed at room temperature on a rotator excluding primary antibody incubation steps. *In vitro*-produced bovine embryos were fixed in 4% paraformaldehyde (Cat. #28906; ThermoFisher Scientific, MA, USA) for 20 min and held in phosphate-buffered saline (PBS; Cat. #AM9625), covered with mineral oil (Cat. #W1503) at 5°C for up to 4 weeks. For immunohistochemistry, embryos were washed 3 × 10 min in washing buffer (WB; PBS + 0.1% Triton X-100) (Cat. #T8787) in 400 µl volumes using AgTech 6-well plates (Cat. #D18) [8]. Embryos were then permeabilized using PBS + 1% Triton X-100 for 30 min followed by a single 10-min wash (WB). Embryos were placed in blocking buffer (BB) WB + 1% BSA (Cat. #A8806) + 10% normal donkey serum compatible with secondary antibody (Cat. #100–151; GemBio, CA, USA) for 2 h followed by a 10-min wash. Overnight incubation with primary antibody (1:300 – independently optimized) was initiated on a rotator at 4°C in an antibody solution (AB) consisting of WB + 1% BSA, in 96-well

plates sealed with parafilm. Embryos were then washed (WB) 3 × 10 min and then 3 × 20 min. Secondary antibody incubation in AB (1:500) was performed in 96 well plates for 1 h at room temperature and protected from light. After incubation, embryos were washed (WB) 3 × 10 min and then 3 × 20 min. Finally, DNA was stained with the addition of Hoechst 33258 (5 µg/ml; Cat. #14530) in PBS for 20 min followed by a 20-min wash (WB).

For imaging, the centers of 75 × 25 mm glass slides (Cat. # 2948; Corning, NY, USA) were affixed with a single binder reinforcement label (Cat. #05721; Avery®, CA, USA) and 9 µl of PBS was added to the center of the reinforcement label (Figure 1). A single embryo was placed in the PBS and a 22 × 22 mm coverslip (Cat. # 2845–22, Corning) was gently lowered. Following imaging using an epifluorescent microscope (confocal may increase image quality), embryos were recovered by gently sliding the coverslip until the reinforcement label was partially exposed and then by floating

METHOD SUMMARY

CRISPR/Cas9-microinjected bovine embryos can be fixed and stored for confirmation of induced mutations in single embryos by using both immunohistochemistry for protein analyses and DNA extraction for sequencing.

the coverslip with the addition of up to 60 μ l of PBS (Figure 1). Embryos were removed with a pipette and placed in a 400- μ l drop of PBS for holding.

DNA extraction of imaged embryos was achieved by placing single embryos in 10 μ l of QuickExtract (Cat. #QE0905T; Lucigen, WI, USA) in an Eppendorf tube on ice. Samples were heated at 65°C for 6 min and 95°C for 2 min using a thermocycler and held at 4°C or stored at -20°C for further processing [6].

Two rounds of PCR were applied to templates. Primer concentration and PCR conditions were optimized for individual experiments. The first round of PCR was achieved in 20 μ l reactions consisting of 10 μ l of GoTaq Hot Start Green Master Mix (2X) (Cat. #M5123; Promega, WI, USA) with the addition of 0.4 μ l (10 μ M) of forward and

reverse primer each and 9.2 μ l of template. The second PCR reaction was identical to the first with the exception of 5 μ l of PCR product produced from the first reaction, nested primers and 4.2 μ l of nuclease-free water. PCR conditions were as follows: 95° for 3 min, followed by 35 cycles of 95° for 30 sec, 56° for 30 sec, 72° for 30 sec and a final extension of 72° for 7 min. Following amplification, 5 μ l of PCR product was loaded onto a 0.9% agarose gel containing EtBr (0.5 μ g/ml) and 0.5 X TBE running buffer (40 mM Tris-Cl, 45 mM boric acid, 1 mM EDTA) and run at 95V for 1 h with a ladder compatible to expected amplicon size. After confirming amplification of a single band (Figure 1), remaining PCR product was purified using QIAquick PCR Purification Kit (Cat. #28104; Qiagen, Hilden, Germany) following manufacturer specifications. Prior

to the final elution step, a single modification was made by adding 10 μ l of nuclease-free H₂O to the center of the column. DNA was quantified using a Nanodrop and approximately 100 ng of DNA with a 260/280 nm absorbance ratio of 1.8–1.9 was submitted for Sanger sequencing. Both DNA extraction and Sanger sequencing of fixed and imaged embryos proved highly efficient and repeatable (83 and 95%, respectively, Table 1).

Herein we report a useful technique for evaluating both protein expression and determining the mutation status of single, fixed bovine embryos following CRISPR/Cas9 microinjection. In addition, we adapted imaging techniques to include the use of a cost-effective and readily available binder reinforcement label as an alternative to traditional adhesive spacers. Furthermore, we

Figure 1. Placement and recovery of fixed bovine embryos for fluorescent imaging followed by DNA recovery for Sanger sequencing. (A) Placement of single embryos in the center of a reinforcement label with phosphate-buffered saline (PBS) on a glass slide with coverslip. Rotation of the coverslip and addition of PBS to recover imaged embryos by aspiration of remaining fluid. (B) Epifluorescent immunohistochemistry images of WT and edited, fixed bovine embryos stained with DAPI (blue) and anti-OCT4 (green) – (sc8628) and held in PBS on a glass slide with coverslip. (C) Nested PCR product from single bovine blastocysts and CRISPR-injected embryos previously fixed and then subjected to immunohistochemistry. (D) Target sequences and respective electropherograms of un-edited bovine blastocysts and edited CRISPR-injected embryos that were fixed, imaged and DNA extracted followed by PCR purification. Scale bar = 100 μ m. WT: Wild-type.

Table 1. DNA amplification and Sanger sequencing efficiency of fixed bovine embryos following immunohistochemistry imaging.

	Imaged	PCR-amplified	Sequenced
No. fixed embryos	23	19	18
Technique	DNA extraction (imaged/amplified)		Sequencing (amplified/sequenced)
Success rate	83%		95%

provide a valuable method to recover sufficient quantity and quality of DNA from single, fixed embryos compatible with sequencing. Collectively, these techniques allow for direct phenotypic and genotypic comparisons and avoid pooled samples with chimeric genotypes potentially confounding data analyses. In addition, the ability to store fixed embryos until further analyses increases experimental flexibility. Finally, these techniques complement the production of gene-edited livestock embryos by allowing for phenotype evaluation and confirmation of mutation status after microinjection, rather than by first transforming cell lines to confirm the desired genotype followed by nuclear transfer, which are both time consuming and technically challenging. In conclusion, we have optimized a method that allows for gene editing confirmation of fixed and stored single embryos by combining both immunohistochemistry and sequencing after CRISPR/Cas9 microinjection of mammalian embryos.

Author contributions

BD designed and performed experiments and prepared the manuscript. MV optimized PCR conditions. TF, SR and GS provided technical guidance and support. PR supervised the study and manuscript preparation.

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Competing & financial interests disclosure

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