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A Rapid In Vivo Bioassay for Developmentally Active Enhancers

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Corresponding Author:	Kerby C. Oberg, MD, PhD Loma Linda University School of Medicine Loma Linda, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	koberg@llu.edu
Corresponding Author's Institution:	Loma Linda University School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Charmaine U. Pira, B.S.
First Author Secondary Information:	
Other Authors:	Charmaine U. Pira, B.S.
	Shelley A. Caltharp, MD, PhD
	Endika Haro, PhD
Order of Authors Secondary Information:	
Abstract:	A potential regulatory sequence (PRS) can be identified by several approaches including conservation analysis and transcription factor specific chromatin immunoprecipitation followed by next generation sequencing (TF ChIP-seq). TF ChIP-seq generates a large data set (TF-regulatome) that includes PRSs and background interactions. These approaches require secondary low throughput validation in model systems that replicate the temporal and spatial specificity of the PRS. In this report, a protocol is described to rapidly validate activity of PRSs in their developmental context. Following selection of candidate PRSs, the associated sequences are isolated, cloned into a GFP reporter construct, and then transfected into chick embryos. For PRSs that are anticipated to be functional during early development, from gastrulation through early organogenesis, whole embryo electroporation (EP) is recommended. With this technique transfection, incubation and monitoring occurs ex ovo during early gastrulation. The entire embryo is transfected and as structures form, they can be monitored live for PRS activity. This ex ovo

	approach supports development up to early limb bud outgrowth. If the functional activity of a PRS is expected to be later in development or if the investigation is to ascertain its activity during the development of a specific organ, targeted regional electroporation (TREP) is used. This approach requires injection of construct DNA directly into the developing organ, precursor or adjacent cavity prior to electroporation. An approach to transfect the presumptive limb bud using TREP is also outlined and illustrated. TREP supports a rapid organ-specific validation of a PRS' activity. Both techniques can also be used to investigate regulatory domains by site directed mutagenesis of the PRS-reporter construct. The chick as a bioassay offers a rapid effective tool for PRS validation, functional domain determination, and further characterization of the regulatory events responsible for development.
Author Comments:	Thanks for the opportunity. Maybe we will meet up at the next EB meeting. All the best, kerby
Additional Information:	
Question	**Response**
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Cover Letter

LOMA LINDA UNIVERSITY
School of Medicine

Dr. Mary Struziak
Editorial Assistant, Developmental Biology Section
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Stuziak;

Thanks for the invitation to submit our protocol on validating enhancer elements via the chick bioassay approach. We are submitting our original protocol description in the manuscript entitled "A Rapid In Vivo Bioassay for Developmentally Active Enhancers" for consideration of publication in JoVE.

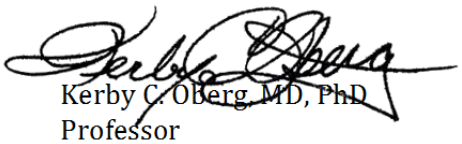
JoVE has published a number of protocols on *in ovo* electroporation, however, our approach assays potential regulatory sequences at various stages of development and includes a method to transfect solids organs and mesodermal tissues with technical tips to enhance targeted delivery. We believe our manuscript describing this approach to assay and validate potential regulatory sequences that function during development will be an important addition to the journals repertoire of protocols and useful for investigators using genomic tools to clarify developmental networks and pathways.

The following authors have participated in developing this protocol: **Charmaine Pira** has performed numerous experiments using this technique. **Charmaine Pira** and **Kerby Oberg** have refined and optimized the protocol. **Shelley Caltharp, Charmaine Pira** and **Kerby Oberg** originally conceived of the approach. Shelley Caltharp performed the conservation analysis on *RTN4/NOGO* locus and generated the associated reporter constructs. **Endika Haro** performed the Lmx1b ChIP-seq analysis. **Charmaine Pira, Endika Haro** and **Shelley Caltharp** generated the PRS and mPRS p*tk*-EGFP constructs.

Suggested reviewers listed below:.
1) Marian Ros – Universidad de Cantabria – email: marian.ros@unican.es
2) Cathy Krull – University of Michigan – email: krullc@umich.edu
2) Susan Mackem – National Cancer Research Institute – email: mackemS@mail.nih.gov
4) Randy Johnson - UT MD Anderson Cancer center – email: rljohnson@mdanderson.org
5) John Fallon - University of Wisconsin – email: jffallon@wisc.edu
6) Marybeth Ezaki - University of Texas Southwestern – email: Marybeth.Ezaki@tsrh.org

All authors have reviewed the manuscript. We have prepared the protocol, list of reagents and equipment, and manuscript as instructed in the authors guide and look forward to your review and comments.

Sincerely,



Kerby C. Oberg, MD, PhD
Professor
Department of Pathology and Human Anatomy,
Divisions of Human Anatomy and Pediatric Pathology

TITLE:
A Rapid *In Vivo* Bioassay for Developmentally Active Enhancers

AUTHORS:
Charmaine U. Pira
Department of Pathology and Human Anatomy
Loma Linda University
Loma Linda, CA, USA
cpira@llu.edu

Shelley A. Caltharp
Department of Pathology
Children's Healthcare of Atlanta
Atlanta, GA, USA
Shelley.Caltharp@choa.org

Endika Haro
Department of Pathology and Human Anatomy
Loma Linda University
Loma Linda, CA, USA
eharo@llu.edu

Kerby C. Oberg
Department of Pathology and Human Anatomy
Loma Linda University
Loma Linda, CA, USA
koberg@llu.edu

CORRESPONDING AUTHOR:
Kerby C. Oberg, MD, PhD

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SHORT ABSTRACT:
With the increased use of transcription factor-specific ChIP-seq technology, techniques to validate potential regulatory regions are critical. The authors describe a rapid chick bioassay to validate the activity of regulatory sequences functioning during development.

LONG ABSTRACT:
A potential regulatory sequence (*PRS*) can be identified by several approaches including conservation analysis and transcription factor-specific chromatin immunoprecipitation followed by next generation sequencing (TF ChIP-seq). TF ChIP-seq generates a large data set (TF-

regulatome) that includes *PRS*s and background interactions. These approaches require secondary low throughput validation in model systems that replicate the temporal and spatial specificity of the *PRS*.

In this report, a protocol is described to rapidly validate activity of *PRS*s in their developmental context. Following selection of candidate *PRS*s, the associated sequences are isolated, cloned into a GFP reporter construct, and then transfected into chick embryos. For *PRS*s that are anticipated to be functional during early development, from gastrulation through early organogenesis, whole embryo electroporation (EP) is recommended. With this technique transfection, incubation and monitoring occurs *ex ovo* during early gastrulation. The entire embryo is transfected and as structures form, they can be monitored live for *PRS* activity. This *ex ovo* approach supports development up to early limb bud outgrowth.

If the functional activity of a *PRS* is expected to be later in development or if the investigation is to ascertain its activity during the development of a specific organ, targeted regional electroporation (TREP) is used. This approach requires injection of construct DNA directly into the developing organ, precursor or adjacent cavity prior to electroporation. An approach to transfect the presumptive limb bud using TREP is also outlined and illustrated. TREP supports a rapid organ-specific validation of a *PRS*' activity. Both techniques can also be used to investigate regulatory domains by site-directed mutagenesis of the *PRS*-reporter construct.

The chick as a bioassay offers a rapid effective tool for *PRS* validation, functional domain determination, and further characterization of the regulatory events responsible for development.

INTRODUCTION:

Evaluation of systems biology by big data sets (*i.e.*, genome-wide) offers new insights and tool sets for the curation of relevant regulatory molecules and pathways.[1,2] Transcription factor-specific chromatin immunoprecipitation followed by next generation sequencing (TF ChIP-seq) generates a genome-wide data set (TF regulatome) of potential regulatory sequences. However, not all of the recovered sites are functionally relevant. The ability to discern signal from noise is a significant limitation of the "omic" data sets.[3]

With an increasing number of completed genomes available for comparison, one approach is to determine whether a potential regulatory sequence (*PRS*) is conserved across species and thereby implicate functional relevance. This approach can accelerate discovery in a variety of disciplines, but is particularly helpful in developmental biology where establishing the basic body plan in vertebrate development is controlled by conserved gene sets.[4] Furthermore, associated regulatory elements are also typically conserved and clustered within introns or associated gene deserts.[5-7] Comparison of the *PRS* with enhancer-associated marks, such as p300 or H327Ac, are additional tools for the prediction of enhancer activity.[8,9] Although these added screening techniques increase the likelihood that *PRS*s are functionally relevant, confirmation is still required.

Timmer and coworkers first reported the use of *in ovo* electroporation as a rapid bioassay for demonstrating enhancer-promoter activity.[10] They injected -*PRS* linked to reporters into chick neural tubes at Hamburger and Hamilton[11] stage (HH) 15-17 using various enhancer-promoter combinations and then followed for enhancer activity. Uchikawa generated an inducible reporter construct with a basal herpes simplex thymidine kinase promoter linked to an enhanced GFP reporter (p*tk*-EGFP).[12] The basal level of activity of this promoter is very low in most tissues allowing for faithful enhancer driven localization of activity. Uchikawa also extended developmental reporting to other tissue involved in early development using a whole embryo electroporation technique at HH 4.[13] Oberg, Pira and colleagues further extended the capacity of *in ovo* electroporation to other tissues at later stages of development.[14,15] Thus, the capacity to evaluate enhancer activity in various tissues through a range of developmental stages has been demonstrated.

In this report, the chick as a bioassay is used at multiple stages and sites to determine the activity of *PRS*s during development. This technique can be used to validate an isolated *PRS* as the target of a hypothesis driven question or to validate several candidates from a large data set before further analysis.

PROTOCOL:

The protocol described below was performed in accordance with Loma Linda University's policy on research involving animals and followed the recommendations within the Federation of Animal Science Societies' (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd ed. and the National Research Council's Guide for the Care and Use of Laboratory Animals. It is important to check with the local institution regarding their requirements for vertebrate animal use approval when using chick embryos since they are not considered as a vertebrate animal within the FASS guide; however, recommendations for appropriate avian embryo euthanasia are described and adhered to in this lab.

1) Construction of the Reporter Vector

1.1) Design primers to the potential regulatory sequence (*PRS*) to contain the restriction endonuclease sequence recognition site for XhoI (CTCGAG) and KpnI (GGTACC). [16]

1.2) Amplify the region by performing polymerase chain reaction (PCR) using genomic DNA template as previously reported.[17,18]

1.3) Clone the PCR product into the p*tk*-EGFP expression vector.[19]

1.3.1) Use the empty p*tk*-EGFP expression vector as a negative control to indicate the basal level of expression within a given tissue. Use the β-actin-driven RFP reporter plasmid as a positive control and an indicator of transfection efficiency.

2) Whole Embryo Electroporation

2.1. Preparations

2.1.1) Prepare filter paper supports. Punch 4 holes in a cloverleaf pattern onto a 2.2 cm x 2.2 cm filter paper using a standard hole punch. Sterilize by autoclaving.

2.1.2) Prepare the albumin-agar culture dish.

2.1.2.1) Dissolve 0.18 g of agar in 30 mL 0.9% sodium chloride on a hotplate, with stirring. Cool in a 42 °C water bath.

2.1.2.2) Extract the thin albumin by first cracking the narrow end of an unincubated egg and removing the eggshell. Pour out the thick albumin and transfer the thin albumin into a sterile 50 mL conical tube using a large bore plastic transfer pipet. Use enough eggs to get 30 mL of thin albumin (~4 eggs). Add 150 µL of 10,000 U/mL penicillin-streptomycin and warm the albumin in a 42 °C water bath.

2.1.2.3 Combine the albumin and agar and mix by swirling. Transfer 2 mL aliquots to a 35 mm Petri dish, gently swirling the dish to ensure even coverage. Allow the agar to set at room temperature for at least 10 min. Store at 4 °C.

2.1.3) Prepare the cloned *PRS* vector cocktail: 1 µg/µL of *PRS* plasmid, 0.3 µg/µL of reporter plasmid, and 0.025% Fast Green FCF stain.

2.2) Protocol for *in vitro* culturing of Hamburger-Hamilton stage (HH) 4 chick embryos

2.2.1) Incubate fertilized White Leghorn eggs at 39° C for 18-19 hr, according to Hamburger-Hamilton chick staging[11].

2.2.2) Spray the surface of the egg with a light mist of 70% ethanol and air-dry.

2.2.3) Crack the egg and empty its content into a 15 cm Petri dish. Carefully remove the thick albumin on the yolk using the blunt end of forceps.

2.2.3.1) If the embryo is on the side of the yolk, roll the yolk around with the blunt end of forceps until the embryo is in the middle of the yolk.

2.2.4) Place the filter paper on the yolk, with the embryo centered. Cut the vitelline membrane at the edges of the filter paper. Lift the filter paper from the yolk.

Note: It is important that the vertical orientation of the primitive streak is maintained so that the embryo does not tear.

2.2.5) Place the filter paper on the albumin-agar plate with the hypoblast (yolk side) up.

Carefully remove excess yolk with the blunt end of forceps. Gently rinse the embryo with warmed Hank's Balanced Salt Solution (HBSS).

2.3) Electroporation Protocol

2.3.1) Position the electrodes and pulled glass capillary needle attached to a syringe under a dissecting microscope. Set the 3 mm round platinum cathode at the base of a ~2 mm deep chamber under the embryo suspension silicone platform. Mount the 2 mm round platinum anode in a micromanipulator and suspend above the silicone platform. The syringe contains mineral oil as the hydraulic fluid.

2.3.2) Add 100-200 µL HBSS into the cathode cavity of the silicone platform. Place the filter paper supporting embryo on the platform with the hypoblast (yolk side) up.

2.3.3) Inject 1 µL *PRS* vector cocktail between the blastoderm and the vitelline membrane using a pulled glass capillary needle. Follow with a small amount of mineral oil to seal the injection point.

2.3.4) Position the anode about 2 mm above the embryo. Drop ~100 µL of HBSS directly over the electrode and embryo. Electroporate with 5 pulses of 5V for 50 msec at intervals of 100 msec.

CAUTION: The electroporator can produce high levels of voltage and current. Observe basic lab safety and common sense to prevent injury. This includes not touching the exposed electrodes with bare hands or materials that can conduct electricity while the electroporator is on. Refer to the equipment manual for other safety precautions and potential hazards.

2.3.5) Return the embryo to the agar-albumin plate, with hypoblast up, and incubate in a humidified chamber.

2.3.6) Clean the cathode after each electroporation by aspirating any remaining liquid and yolk. Clean the anode by wiping with a lab tissue.

3) Targeted Regional Electroporation: Neural Tube of HH10 Chicken Embryo

3.1) Protocol for windowing the egg and staining the embryo

3.1.1) Incubate fertilized White Leghorn eggs at 39 °C for 33-38 hr, according to Hamburger-Hamilton chick staging[11].

3.1.2) Spray a light mist of 70% ethanol onto the eggs and air-dry.

3.1.3) Place the egg on its side on an egg holder. Using a dissecting probe, poke a small hole at the blunt end of the egg. Insert a syringe with an 18 gauge needle through this hole at a

downward angle and withdraw 1-2 mL of albumin, taking care not to remove any yolk.

3.1.4) Without breaking the underlying shell membrane, poke a shallow hole on the top side of the egg. Carefully remove small portions of the eggshell with the dissecting probe, until ~0.5 cm diameter of the underlying membrane is exposed. Break the shell membrane by pushing down on it with the dissecting probe.

3.1.5) Cover the hole with a strip of tape and cut the hole bigger, to ~2 cm diameter, using small scissors.

3.1.6) Under a dissecting microscope, identify the blood island surrounding the embryo and add 6 µL of neutral red solution to the middle of this blood island. Wait 2-3 min.

3.1.7) Carefully remove the vitelline membrane overlying the embryo using a tungsten needle.

3.2) Electroporation Protocol

3.2.1) Align the two electrodes in parallel with a fixed inter-electrode distance of 4 mm and mount on a micromanipulator. Position the electrodes on the yolk membrane so that the embryo is centered between the electrodes.

3.2.2) Inject ~0.1 µL *PRS* vector cocktail into the neural tube lumen of the embryo. Add 1-2 drops of 1X phosphate buffered saline (PBS) using a transfer pipet. Reposition the electrodes so that they touch the yolk membrane, if necessary.

3.2.3) Apply 5 pulses of 20 V each lasting 50 msec at 950 msec intervals. Add 1 drop of 1X PBS and remove electrodes.

3.2.4) Seal the egg with a piece of transparent tape and return to the egg incubator.

3.2.5) Clean electrodes after each electroporation in bleach, RNase-free water, and 1X PBS to remove residual yolk.

4) Targeted Regional Electroporation: Lateral Plate Mesoderm of HH14 Chicken Embryos

4.1) Electroporation Protocol

4.1.1) Incubate fertilized White Leghorn eggs at 39° C for 50-53 hr, according to Hamburger-Hamilton chick staging[11].

4.1.2) Open and stage the embryos as described above in **in Steps 3.1.2 to 3.1.7**

4.1.3) Using a tungsten needle, make a slit onto the yolk membrane outside the periphery of the blood vessel at the tail end of the embryo.

4.1.4) Slide the cathode rod into the yolk through this slit. Position the cathode parallel to and along the length of the embryo. Do not place the cathode directly under the lateral plate mesoderm.

4.1.5) Inject ~0.2 µL *PRS* vector cocktail into the intraembryonic coelom at the level of somite 18, followed by a small amount of mineral oil. The DNA will travel anteriorly and posteriorly along the coelom.

4.1.6) Position the cathode directly under the lateral plate mesoderm, spanning somites 15-20. Position the anode above the lateral plate mesoderm with an inter-electrode distance of ~2.5 mm. Add 3-5 drops of 1X PBS so as to submerge the anode. Adjust the electrodes so that they are not touching the embryo.

4.1.7) Apply 3 pulses of 8 V, 60 msec in duration at 50 msec intervals. Remove cathode from the yolk through the slit.

4.1.8) Add 40 µL 2,500 U/mL of penicillin/ streptomycin. Seal the egg with transparent tape and return to the egg incubator.

4.1.9) Clean electrodes after each electroporation in bleach, RNase-free water, and 1X PBS to remove residual yolk.

5) Fluorescence analysis

5.1) Whole embryo electroporation

5.1.1) Using forceps, lift the filter paper from the plate and replace the embryo onto the same plate with the hypoblast down. Position the embryo away from residual yolk and cloudy portions of the agar-albumin plate. Alternatively, place the embryo in a new plate for imaging.

5.1.2) Examine the embryos with a fluorescence microscope to visualize transfection efficiency and *PRS* activity 6-48 hr after transfection. To optimize fluorescence intensity, visualize the embryos under high magnification.

5.2) Targeted regional electroporation (TREP).

5.2.1) Carefully dissect the embryo from the egg by cutting the yolk membrane around the embryo with microscissors and transfer to a 35 mm dish containing 1X PBS.

5.2.2) Under a dissecting microscope, remove the extraembryonic membrane with microscissors and forceps.

5.2.2.1) Keep the microscissors flat and parallel to the embryo to prevent accidentally cutting

the embryo. Do not pull or tear the membranes with forceps as you might also tear the embryo.

5.2.3) Visualize the embryo in a new 35 mm dish with 1X PBS under bright field and fluorescence microscopy. Use high magnification (40x) to optimize fluorescence intensity.

5.2.3.1) At high magnification, stitch the images together using an image-editing software to generate the complete image of the targeted tissue.

REPRESENTATIVE RESULTS:

The p*tk*-EGFP vector provides an inducible reporter with low levels of background activity in the developing chick embryo. The vector links the Herpes Simplex virus minimal thymidine kinase promoter (p*tk*) to an enhanced green fluorescent protein (EGFP) reporter (Figure 1A). The potential regulatory sequence is cloned into the multiple cloning site (MCS) upstream of the promoter. The construct is then transfected into the chick embryo by electroporation. Transfection efficiency is determined by co-electroporation with a β-actin promoter-driven RFP reporter plasmid (Figure 2D). RFP can be detected within 6 hr of transfection indicating the lag time needed before enhancer activity can be assayed with certainty. When assaying for activity during early development, whole embryo electroporation *ex ovo* at HH 4 during gastrulation is used (Figure 1B). Activity can then be localized and followed through gastrulation and basic body plan formation, up until about stage HH 16 or 17 just prior to wing outgrowth.

Whole embryo electroporation was used to assay potential regulatory sequences (*PRS*) associated with the *RTN4/NOGO* gene. A *PRS* 88.5 kb upstream of *RTN4/NOGO* demonstrated enhancer activity in the neural tube (Figure 2). The product of *RTN4/NOGO*, NOGO-A, is associated with neurite outgrowth inhibition. *NOGO-A* is also expressed within the neural tube coincident with the activity of the *PRS*. To assay the activity of this NOGO-A associated enhancer element (*NAEE*) at later stages of development, targeted regional electroporation (TREP) was used to transfect the neural tube at HH 10 (Figure 1C). TREP demonstrated that *NAEE* activity became restricted to the cranial spinal cord by HH18 (Figure 2N).

There are three predicted binding sites within this short 125 bp, enhancer; POU1F1, HNF3B, and SOX. Site-directed mutagenesis of the SOX binding site silenced enhancer activity suggesting that a SOX transcription factor is required for enhancer activity (Figure 2 I & J). The ZPA regulatory sequence (ZRS) has been described as the most distant regulatory enhancer located 1Mb upstream of *Sonic hedgehoh* (*Shh*).[20] Thus, of the genes within the vicinity (~1 Mb) of the *NAEE*, only *RTN4/NOGO* is expressed within the neural tube. Thus, *RTN4/NOGO* is the most likely target of *NAEE* regulation.

In addition, transcription factor-targeted ChIP-seq data (reported as genomic intervals or peaks) are considered potential regulatory sequences. Genomic-wide sites of Lmx1b binding within the developing murine limb bud were analyzed at embryonic day 12.5 (e12.5). The genomic intervals were screened for conservation and a *PRS* 60 kb upstream of *Lmx1b* itself was identified (Figure 3A). To validate that this *PRS* could function as an enhancer during limb

development, a p*tk*-EGFP reporter construct was transfected into the presumptive chick limb using TREP (Figure 1D). After 48 hr of incubation, the emerging limb bud (HH 24; developmentally similar to e12.5) demonstrated enhancer activity restricted to the dorsal limb mesoderm corresponding to the expression domain of *Lmx1b* (Figure 3D & E). Enhancer activity was abolished by mutation of the predicted Lmx1b binding site, implying a requirement for Lmx1b binding for enhancer activity (Figure 3H).

Figure 1: Reporter Construct and Electroporation Setup
A) The p*tk*-EGFP vector illustrating the multiple cloning site (MCS), the thymidine kinase promoter (p*tk*) linked to the enhanced green fluorescent protein (EGFP) reporter. Potential regulatory sequences (*PRS*) are cloned into the MCS just upstream of the minimal promoter. The *PRS*-p*tk*-EGFP can then be transfected into chick embryos and assayed for activity using one of the following three approaches. B) Whole embryo electroporation. A Hamburger-Hamilton stage (HH) 4 embryo is mounted on a filter paper support and placed hypoblast up on a silicone platform encasing the cathode (-). The DNA is injected into the space between the vitelline membrane and the epiblast and the anode (+) is suspended above the embryo (PP=primitive pit, PS=primitive streak). C) Targeted regional electroporation of the neural tube. DNA is injected into the lumen of the neural tube of HH 10 chicken embryo. Paired parallel electrodes (+, -) are placed flanking the embryo. D) Targeted regional electroporation of the lateral plate mesoderm of HH 14 chicken embryos. DNA (*PRS*-p*tk*-EGFP) is injected into the intraembryonic coelom underlying the lateral plate mesoderm between somite levels 15 and 20 (black squares). The cathode (-) is placed underneath the embryo and the anode (+) is placed above the embryo, as shown in the transverse section at the level indicated in D with a black line (inset) (Cathode – colored black, Anode – colored red, Injected DNA – colored green)

Figure 2: Activity of the *NOGO-A*-Associated Enhancer Element (*NAEE*) During Development.
A) Schematic of the potential regulatory sequence upstream of the *RTN4/NOGO* gene that encodes for NOGO-A and its reporter construct. p*tk* – basal thymidine kinase promoter B) *in situ* hybridization (ISH) for *NOGO-A* mRNA at HH 10. C) Bright-field light of a HH 10 embryo transfected with the *NAEE*-p*tk*-EGFP reporter. D) Co-transfection of the embryo with an RFP reporter plasmid demonstrates transfection efficiency. E) Activity of the *NAEE* sequence within the neural tube (NT). F) Boxed region from "E" magnified. *NAEE* activity (E) co-localizes with *NOGO-A* expression (B). G) Bright-field of a similarly staged embryo assaying *NAEE* activity with the SOX binding site mutated (*mSOX NAEE*) showing loss of activity (I). J) Boxed region in I magnified. H) RFP is diffuse indicating good transfection. K) *NOGO-A* expression in a later stage (HH 18) embryo. L) Bright-field of the HH 18 embryo. N) Enhancer activity is accentuated in the spinal cord whereas RFP is detected throughout the neural tube (M). Scale bars in C, G and L are 1 mm.

Figure 3: Validation of an *Lmx1b*-Associated Regulatory Element (*LARE*) Identified by ChIP.
A) Schematic of the conserved sequence found by Lmx1b-specific ChIP-seq and its reporter construct (p*tk* – basal thymidine kinase promoter). At HH 24 (48 hr after transfection of the presumptive limb mesoderm), the limb is examined. B) Morphology of the HH 24 limb bud with bright-field light (scale bar = 500 μm). C) Distribution and transfection efficiency is

demonstrated by an RFP reporter plasmid. D) Activity of the conserved ChIP-identified genomic interval is evident and localized to dorsal mesoderm corresponding to the expression domain of *Lmx1b* (E, *in situ* hybridization to *Lmx1b* mRNA). F) Bright-field of an HH 24 limb transfected with the *LARE* containing a mutated Lmx1b binding site (*mLARE*; scale bar = 500 µm). G) Diffuse distribution of RFP indicating transfection efficiency. H) Loss of activity when the Lmx1b binding site is mutated.

DISCUSSION:

This report describes a bioassay to validate potential regulatory sequences active during development. The approach incorporates the use of a reporter construct, transfection via electroporation and chicken embryos. The process, from sequence determination to validation of activity, can typically be completed within two to four weeks. A critical aspect of this protocol is the efficient transfection of construct into the target tissue. Several steps ensure efficient transfection. The use of endotoxin-free plasmids minimizes post-operative deformation and embryonic death. Cleaning electrodes well after each EP optimizes and standardizes transfection. The voltage and pulses of the electroporator may also need to be adjusted to optimize transfection yet minimize EP related tissue damage. These parameters, once established should be consistently used. For whole embryo EP, removing as much of the yolk from the embryo and filter paper as possible optimizes current flow and keeps the embryo from sagging or tearing from the filter paper. Removing the yolk also makes it easier to visualize the embryo and *PRS* activity during evaluation.

For targeted regional electroporation (TREP), injection of DNA into a cavity is ideal, since the cavity can retain the concentrated DNA during the time between injection and electroporation. Every attempt should be made to keep this interval short irrespective of the target, but a restricted space is helpful. When an associated cavity or restricted space is not available, a cavity or bubble within the target tissue should be created. For example, Oberg and co-workers created a cavity within limb bud mesoderm by injecting a bolus of DNA followed by a bubble of mineral oil.[14] Retention of DNA by this approach promoted efficient transfection of limb bud mesoderm. If direct tissue contact by the electrodes is necessary to direct current to the target tissue, insulate the electrodes as described by Oberg and co-workers to minimize short circuits that can reduce transfection efficiency.[14] Be aware that direct contact will also cause some local tissue damage. If TREP of an organ has yet to be described, the approach should be optimized first with the RFP construct to ensure effective transfection of the targeted tissue with minimal tissue damage. The major limitation of this approach is the transfection of organs that form in more advances stages of development or are physically difficult to target within the embryo such as the metanephric kidney.

A number of *in vitro* and *in vivo* approaches have been used to validate potential regulatory sequences. Validation of an enhancer element can be accomplished in a relevant *in vitro* cell line.[21] Although this approach can be useful, cell lines are immortal and out of context from their original derivation and thus, may not represent *in vivo* activity. Even primary cells grown in culture are out of context to their *in situ* relationships and rapidly lose some of their differentiated features.[22,23] For a transcription factor that has precise spatial and temporal

expression during development, context is critical for functional validation. Analysis of enhancer activity in transgenic mice follows a protocol similar to what is described in this report with generation of a reporter construct, typically using the LacZ reporter, although GFP has also been described.[24,25] The mouse transgenic approach requires the injection of the construct into mouse embryonic stem cells, typically injecting 100-300 cells for recovery 3-5 embryos, 3-7 week after construct generation, and costs of around $2500 per construct. Furthermore, since evaluation occurs post-harvesting only one developmental stage can be examined per embryo. Thus, using the mouse transgenic approach to evaluate developmentally active vertebrate enhancers is limited by embryonic stage, cost, time and efficiency. The zebrafish model is a more assessable and accessible model for enhancer activity but is also a vertebrate model that is more divergent from human than either mouse or chicken.[7] Thus, for the analysis of limb development related enhancers, the chick or mouse models would be more informative. The zebrafish model also requires establishing a fish facility to breed and maintain a colony, which is not a trivial undertaking.

The chick is a common well-studied vertebrate developmental model for several major organ systems including brain, heart and limbs. Fertile eggs are readily available and require only an avian incubator for development. Similar to the zebrafish model, assessment of enhancer activity in chick embryos is rapid, within 6 hours after transfection, and as in zebrafish, enhancer activity can be followed through multiple stages of development. The most expensive item required to setup up a chick bioassay system for enhancer activity in most labs is the electroporation unit that is presently around $14k, which can be used for hundreds of transfections and bioassays of potential regulatory sequences. Considering the cost of mouse transgenics or establishing a fish facility, the chick bioassay is quite cost effective. Overall, the chick bioassay as described provides a rapid system for validating enhancers active during vertebrate development. The approach can be used to supplement high throughput techniques to confirm functional relevance of data sets. Alternatively, this approach can be used to validate targets extracted from genomic data sets that are to be used in hypothesis-driven, mechanistic investigations.

ACKNOWLEDGEMENTS:
The authors would like to thank the members of the Soriano lab for critical review of this technique. The authors would also like to thank Dr. Uchikawa for the kind gift of the p*tk*-EGFP plasmid. Funded in part by grants from the LLU Pathology Research Endowment, National Organization of Rare Diseases, and from the NICHD (HD39421).

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1 (Preferred)

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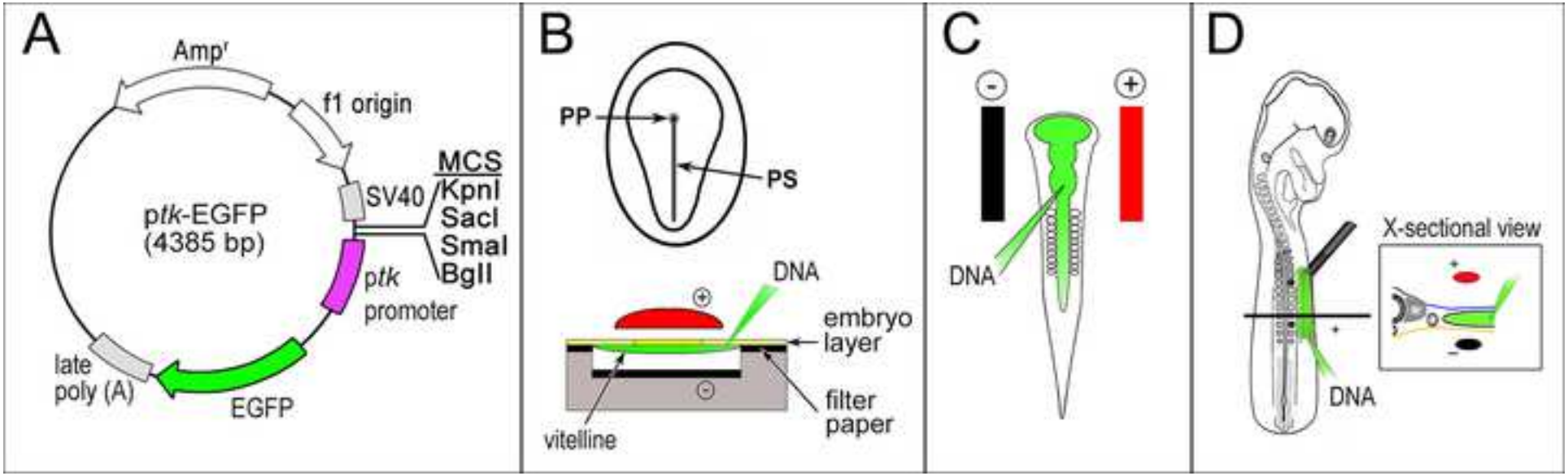

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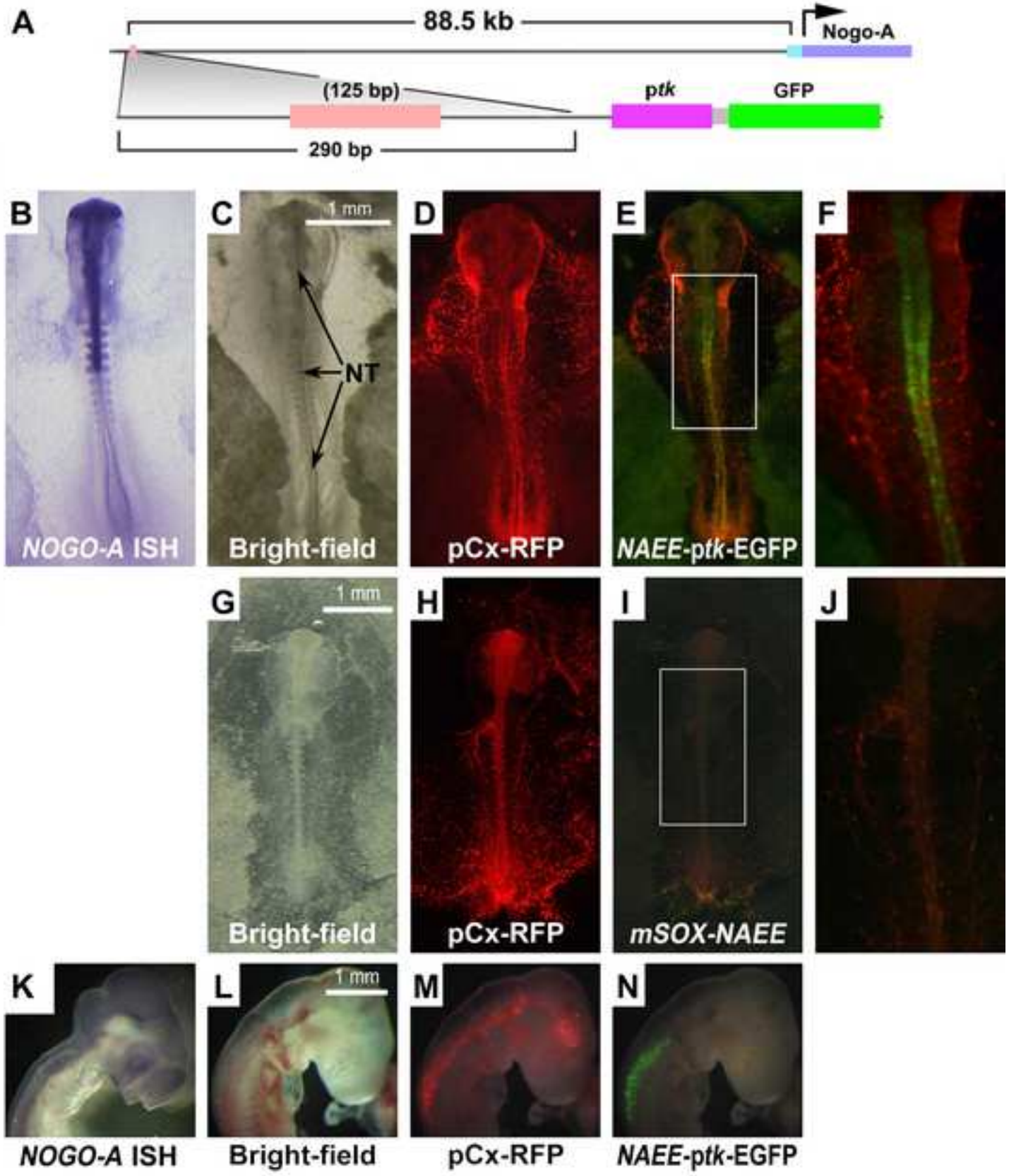

Figure 3

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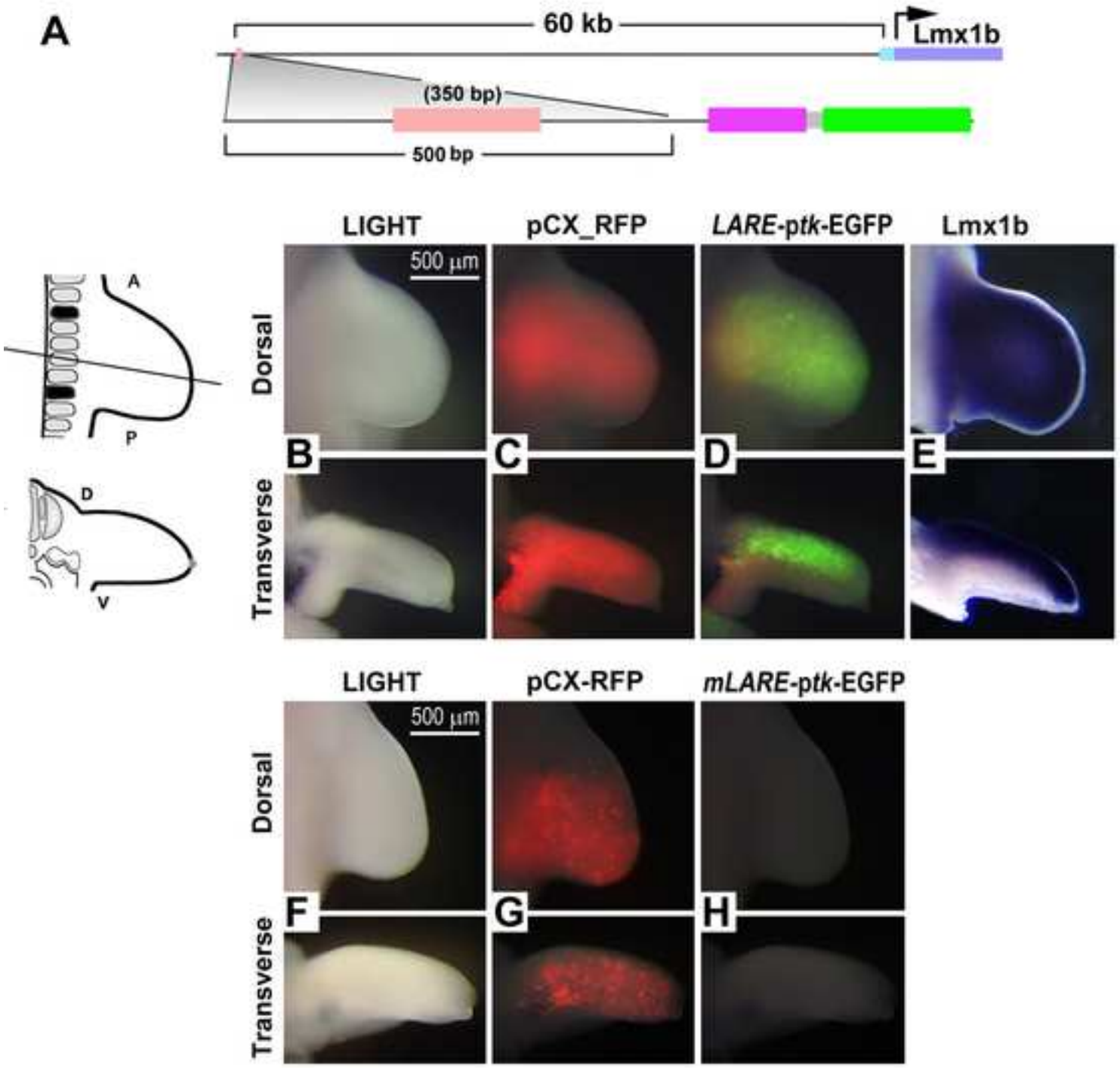

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Bacto-Agar	BD Biosciences	204010	
Sodium chloride	Sigma	S9888	
Hank's Balanced Salt Solution (HBSS)	Sigma	H6136	Hank's Balanced Salt Solution (HBSS),
Penicillin-streptomycin	Life Technologies	15140-122	
Ethanol 200 proof			dilute to 70%
Endofree Maxiprep Plasmid Kit	Qiagen	12362	EndoFree Plasmid Maxi Kit
White Leghorn fertilized eggs	Chino Valley Ranchers		
Whatman Grade 4 filter paper	GE Healthcare Life Sciences	1004-240	filter paper support (a range of grade
50ml conical tube	VWR	21008-242	
35mm petri dish	VWR	627161	Sterile Petri Dishes, 35W x 10H mm
15cm petri dish	VWR	82050-600	Sterile Petri Dishes, 145W x 20H mm
Transfer pipet	VWR	414004-042	
Capillary tube	World Precision Instruments	1B100F-6	
Terasaki dish	VWR	82050-706	dish from which to aspirate DNA
mineral oil			
Standard hole punch			
Hotplate stirrer			
Stir bar			
Water bath			
Round Platinum 3mm Petri dish electrode	Protech International Inc	CUY700-P3E	electrode for whole embryo electrop
Round Platinum 2mm cover electrode	Protech International Inc	CUY700-P2L	electrode for whole embryo electrop
Humidified chamber			
Dissecting microscope			
Dissecting fluorescence microscope			
Forceps			`
Standard surgical scissors			
CUY21-EDIT Square Wave Electroporator	Protech International	CUY21-EDIT	electroporator

Micropipette	Hamilton Company		
Micromanipulator			
Egg holder (nest)			modeling clay shaped on a 10cm pet
Dissecting probe			to window the egg
Parallel Fixed Needle Electrode	Protech International Inc	CUY610-P4.4	4mm Gap & Platinum 4mm Tip, electı
Tungsten wire	Omega Engineering, Inc	W5W26-010	Tungsten 5% Re vs. Tungsten 26% Re,
Needle holder			
Platinum Z-Shape Blunt Needle Electrod	Protech International Inc	CUY611-P7.4	7mm Length x 4mm Exposed Tip, elec
18 gauge needle			
10cc syringe			
Scotch transparent tape	Office Depot	305324	
Neutral red solution 0.25%	Fisher Scientific	N12925	to stain the embryo
Dulbecco's Phosphate Buffered Saline	Sigma aldrich	D5773	Dulbecco's Phosphate Buffered Saline
Household bleach			for electrode cleaning
Fast green FGF (0.25%)	Specrtum Chemical Mfg Corp	FA105	
Predicted regulatory region Plasmid			
Reporter pasmid	Several options		The p*tk* EGFP was a kind gift from Uch
		included in Endofree	
TE buffer	Qiagen	Maxiprep Plasmid Kit	for dilution of DNA

Without sodium bicarbonate

s are ssufficent to provide stability during growth)

oration

oration

ri dish

rode for electroporation of neural tube
, 0.010, unsheathed, used to make fine cuts on embryo

ctrode for electroporation of lateral plate mesoderm

e, Without sodium bicarbonate

nikawa and Kondoh, but Adgene has a p*tk* RFP that could be used for the reporter and pCAG-GFP for the transfection control.

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Dr. Nam Nguyen,
Science Editor, and
Aleksandra Jachtorowicz
Deputy Director of Editorial - Life Sciences
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Dear Drs Nguyen and Jachtorowicz;

Thank you for the editorial comments and we are especially grateful for the reviewers insight and suggestions. The manuscript will be better because of their input.
Please see the attached responses to each editorial concern and each reviewer concern and comment.

Also note that all of the authors have reviewed the revised manuscript, figures and comments.

Also, congratulations on receiving your first impact factor. Your hard work is paying off and I am sure that the impact will continue to climb in this highly interactive environment.

Sincerely,

Kerby C. Oberg, MD, PhD
Senior Administrative Professor
Department of Pathology and Human Anatomy,
Divisions of Human Anatomy and Pediatric Pathology

Responses to Editor and Reviewer comments/critiques:

1. Please proofread the manuscript for spelling or grammar issues:
We have thoroughly reviewed the manuscript for spelling and grammar issues and made corresponding corrections.

2. Please include an ethics statement indicating that the protocol follows the animal care guidelines of your institution.
We have added an ethics statement as requested – lines 108 -115

3. Please the protocol text is a hard 2.75 page limit of highlighted protocol text for filming.
The highlighted protocol has been reevaluated and reduced as requested.

4. Some additional details are required:
•5.2.1 - What steps are involved with this process? Any tips?
•5.2.3 - What magnification should be used?
The additional details have been addressed in the protocol. Tips see 5.2.2.1 lines 355-357. Magnification for different steps see lines 343-344, 349-350, and 352

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this paper, the Authors describe a rapid, effective and cheap system for validating regulatory regions, based on transfection of chicken embryos with reporter plasmids. This system is particularly useful to study developmentally-regulated enhancers.
Thank you

Major Concerns:
1. The protocol is well described, but probably not easy to understand for a lab where there is no expert in chicken development. The video associated with the manuscript will be essential to acquire these techniques.
We have carefully evaluated the protocol with this concern in mind and have revised accordingly while keeping within the 2.75 page limit.

2. Considering the inter-laboratory variability, should each lab set up the best electroporation conditions (voltage, pulses)? The Authors should comment on the setting up of the electroporation conditions.
Appreciate the comment. We added our approach to optimizing the EP parameters for each embryonic stage or condition — see lines 463-465

Minor Concerns:
1. It would be useful to mention if the plasmids are available (Addgene, which laboratories can provide them).
The p*tk*EGFP plasmid was a gift from M. Uchikawa and H. Kondoh. Addgene does not have tk-EGFP but they have variants (tk-RED) and they refer to other companies that offer additional variant reporters (eg., Thermo Scientific: luciferase). A note was added to the acknowledgement section and a comment in the reagent excel file.

2. The cloning step is a standard procedure, the list of reagents for this step may be excluded from the text (besides the endofree maxiprep that is essential, as mentioned in the discussion, to reduce the embryo damages).
The common molecular biology reagents (enzymes, etc) have been deleted from the materials list in the excel file as requested, except for the endotoxin free maxiprep kit which is an essential element of the protocol. Mineral oil for the oil bubble technique was also added to the materials list.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Given the emerging high-throughput methods for enhancer discovery, it will be useful to identify techniques for rapid validation of enhancer elements. The authors describe rapid chick bioassays for in vivo validation of putative regulatory elements. They discuss whole animal and targeted regional electroporation methods of introducing putative regulatory elements which are tested for their ability to drive reporter expression during chick development. The method is well-written, and the video promises to be informative. The authors have done a very nice job, and I have only a handful of suggestions for improvements:
Thank you

Major Concerns:
It would be good to suggest controls -- other than mutated enhancers shown in your representative data -- that might be easier to construct. Also, you mention the RFP construct positive control in line 424, but it might be good to mention this up front. I would add discussion of both negative and positive controls in 1).
In 1.4 of the protocol we indicate that the empty p*tk*-EGFP expression is used as a negative control to demonstrate basal activity of the plasmid and the beta-actin-driven RFP is used as a positive control and indicator of transfection efficiency. (Lines 127-129)

Minor Concerns:
Would it be appropriate to include an ethics statement and an indication that those who use this method should seek vertebrate animal approval?
An ethics statement regarding the use of vertebrate animals and the need to inquire about whether approval is needed for embryos has been included. Lines 108-115

Are there any safety precautions to be taken for the electroporation procedure?
Thanks for noting this – we have added a precautionary comment regarding the use of the electroporator. See step 2.3.4.1 in the protocol lines 218 - 221

Is a dissecting scope used/helpful during the embryo preparation?
Added "under dissecting microscope" when used throughout the protocol – see lines 197-198, 253 and 352.

You may wish to consider demonstrating the oil bubble method (line 419) in your video protocol, as it might be useful to see this step. It looks tricky.
The oil bubble was included in the video protocol – see lines 207-210 and lines 307-309

Line 102: TO validate
Thank you, the "to" was added in front of the validate in line 104

Line 389: ptk should be italicized; kinase instead of kiniase
Thank you, these have been corrected – see line 429 and 445

Line 447: maintain instead of maintaining
Thank you this has been corrected – see line 504

Fig. 1D: What is the pink area in the X-sectional view?
The pink area is illustrating a somite in x-section and extraneous to the lateral plate being electroporated. I have removed the color to minimize it distracting readers from the point of the illustration. I have also included an alternate image that labels the somite (so) and neural tube (nt) to improve clarity of the structures illustrated, but I think these are not necessary if the color has been removed. I prefer the first image - Fig 1mA.tif. If the alternate is preferred we will need to modify the legend to annotate these additional terms.

Fig. 2 E, M and N: These printed pretty dark, so you might want to brighten these images.
As requested, we have brightened images M &N (and also K & L within the same panel). We have also brightened E also with a slight increase in contrast.