# GENE-SWitCH

**The regulatory GENomE of SWine and CHicken: functional annotation during development**

# Protocol WP1
# Strand-Specific PIG and CHICKEN RNA Library Preparation and NovaSeq Sequencing

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|---|---|
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# 1 Summary

GENE-SWitCH aims at identifying functional elements located in the genomes of pig and chicken working on seven different tissues at three different developmental stages. It requires a collection of samples corresponding to the selected tissues and developmental stages with associated metadata describing accurately the samples and the sampling process.

The seven tissues analysed in GENE-SWitCH are:

- Cerebellum
- Lung
- Kidney
- Dorsal skin
- Small intestine
- Liver
- Skeletal muscle

The three developmental stages are:

- Early organogenesis (E8 chick embryo and D30 pig foetuses)
- Late organogenesis (E15 chick embryo and D70 pig foetuses)
- Newborn piglets and hatched chicks

For each species and each developmental stage, 4 biological replicates (2 males and 2 females) are sampled. The 84 chicken samples were prepared according protocol: https://data.faang.org/api/fire_api/assays/ROSLIN_SOP_GENESWITCH_WP1_CHICK_EXTRACTION_DNA_RNA_20201111.pdf and pig samples according protocol below: https://data.faang.org/api/fire_api/assays/INRA_SOP_GENESWITCH_WP1_PIG_EXTRACTION_DNA_RNA_20201111.pdf.

All samples were send to Genewiz subcontractor for further samples processing.

## 2. Protocol description

RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA).

RNA sequencing library preparation was prepared using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). For polyA selection, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented. First strand and second strand cDNA were subsequently synthesized. The second strand of cDNA was marked by incorporating dUTP during the synthesis. cDNA fragments were adenylated at 3'ends, and indexed adapter was ligated to cDNA fragments. Limited cycle PCR was used for library amplification. The dUTP incorporated into the cDNA of the second strand enabled its specific degradation to maintain strand specificity. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA).

The sequencing libraries were multiplexed and clustered on the flowcell. After clustering, the flowcell was loaded on the Illumina NovaSeq 6000 instrument according to manufacturer's instructions. The samples were sequenced using a 2x150 Pair-End (PE) configuration. Image analysis and base calling were conducted by the NovaSeq Control Software v1.6 on the NovaSeq instrument. Raw sequence

data (.bcl files) generated from Illumina NovaSeq was converted into fastq files and de-multiplexed using Illumina bcl2fastq program version 2.20. One mismatch was allowed for index sequence identification.

The detailed protocol for library production can be downloaded using the following link:

https://international.neb.com/protocols/2017/02/07/protocol-for-use-with-purified-mrna-or-rrna-depleted-rna-and-nebnext-ultra-ii-directional-rna-library-prep-kit-for-illumina-neb-e7760-e7765

and is also reproduced below:

**Protocol for use with Purified mRNA or rRNA Depleted RNA and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, #E7765)**

**Symbols**

*This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.*

*This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.*

*Colored bullets indicate the cap color of the reagent to be added*

**RNA Integrity:**

RNA Integrity Number (RIN) is computed using ribosomal RNA (rRNA) amount in the sample. If rRNA is removed by any method, the RIN value should not be used to evaluate the integrity of the RNA sample. In this case, we recommend that the fragmentation time is empirically determined if the RNA sample is suspected to be low quality. **The following recommendation apply to the total RNA samples only.**

Assess the quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). RNA with different RIN values require different fragmentation times or no fragmentation at all.

For intact (RIN > 7) or partially degraded RNA samples (RIN = 2 to 7) follow the library preparation protocol in Section 4 (current Section). See Table 4.1.1 for the recommended the fragmentation times.

For highly degraded samples (RIN = 1 to 2) (e.g. FFPE), which do not require fragmentation, follow the library preparation protocol in Section 5.

**RNA Sample Requirements:**

The RNA sample should be free of salts (e.g. $Mg^{2+}$, or guanidinium salts), divalent cation chelating agents (e.g. EDTA or EGTA) or organics (e.g. phenol or ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all

traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation.

**Input Amount Requirement**
1 ng – 100 ng purified mRNA or rRNA depleted RNA that is **quantified after the purification**. RNA should be DNA free in up to 5 µl of Nuclease-free Water, quantified by Qubit Fluorometer and quality checked by Bioanalyzer.

The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A for recommended fragmentation times and size selection conditions.

This protocol has been optimized using Universal Human Reference Total RNA.

### 2.1. RNA Fragmentation and Priming

**RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.1.1.**

2.1.1. Assemble the fragmentation and priming reaction **on ice** in a nuclease-free tube by adding the following components:

| FRAGMENTATION AND PRIMING MIX | VOLUME |
|---|---|
| Purified mRNA or rRNA Depleted RNA | 5 µl |
| (lilac) NEBNext First Strand Synthesis Reaction Buffer | 4 µl |
| (lilac) Random Primers | 1 µl |
| **Total Volume** | **10 µl** |

2.1.2. Mix thoroughly by pipetting up and down ten times.

2.1.3. Place the sample in a thermocycler and incubate the sample at 94°C following the recommendations in Table 2.1.1 below for fragment sizes ~200 nt.

Table 2.1.1 Suggested fragmentation times based on RIN value of RNA input.

| RNA TYPE | RIN | FRAG. TIME |
|---|---|---|
| Intact RNA | > 7 | 15 min. at 94°C |
| Partially Degraded RNA | 2–6 | 7–8 min. at 94°C |

**Note: Refer to Appendix A for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A only apply for intact RNA.**

2.1.4. Immediately transfer the tube to ice and proceed to First Strand cDNA Synthesis.

### 2.2 First Strand cDNA Synthesis Reaction

2.2.1. Assemble the first strand synthesis reaction **on ice** by adding the following components to the fragmented and primed RNA from Step 2.1.4:

| FIRST STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| Fragmented and primed RNA (Step 4.1.4) | 10 µl |
| (brown) NEBNext Strand Specificity Reagent | 8 µl |
| (lilac) NEBNext First Strand Synthesis Enzyme Mix | 2 µl |
| **Total Volume** | **20 µl** |

2.2.2. Mix thoroughly by pipetting up and down ten times.

2.2.3. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows:

**Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2.**

Step 1: 10 minutes at 25°C
Step 2: 15 minutes at 42°C
Step 3: 15 minutes at 70°C
Step 4: Hold at 4°C

2.2.4. Proceed directly to Second Strand cDNA Synthesis.

### 2.3. Second Strand cDNA Synthesis

2.3.1. Assemble the second strand cDNA synthesis reaction **on ice** by adding the following components to the first strand reaction product from Step 2.2.4.

| SECOND STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| First Strand Synthesis Product (Step 2.2.4) | 20 µl |
| (orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X) | 8 µl |

| SECOND STRAND SYNTHESIS REACTION | VOLUME |
|---|---|
| (orange) NEBNext Second Strand Synthesis Enzyme Mix | 4 µl |
| Nuclease-free Water | 48 µl |
| **Total Volume** | **80 µl** |

2.3.2. Keeping the tube on ice, mix thoroughly by pipetting the reaction up and down ten times.

2.3.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C or off.

**2.4. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads**

2.4.1. Vortex SPRIselect beads or NEBNExt Sample Purification Beads to resuspend.

2.4.2. Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.4.3. Incubate for 5 minutes at room temperature.

2.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **(Caution: do not discard beads)**.

2.4.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.4.6. Repeat Step 2.4.5 once for a total of 2 washing steps.

2.4.7. Air dry the beads **for up to 5 minutes** while the tube is on the magnetic rack with lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

2.4.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

2.4.9. Remove 50 µl of the supernatant and transfer to a clean nuclease free PCR tube.

**Note: If you need to stop at this point in the protocol, samples can be stored at –20°C.**

### 2.5. End Prep of cDNA Library

2.5.1. Assemble the end prep reaction on ice by adding the following components to second strand synthesis product from Step 4.4.9.

| END PREP REACTION | VOLUME |
|---|---|
| Second strand cDNA Synthesis Product (Step 4.4.9) | 50 µl |
| (green) NEBNext Ultra II End Prep Reaction Buffer | 7 µl |
| (green) NEBNext Ultra II End Prep Enzyme Mix | 3 µl |
| **Total Volume** | **60 µl** |

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

2.5.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

**Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**

2.5.3. Incubate the sample in a thermal cycler with the heated lid set at ≥ 75°C as follows:
30 minutes at 20°C
30 minutes at 65°C
Hold at 4°C

2.5.4. Proceed immediately to Adaptor Ligation.

### 2.6. Adaptor Ligation

2.6.1. Dilute the (red) NEBNext Adaptor prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the diluted adaptors on ice.

| PURIFIED RNA | DILUTION REQUIRED |
|---|---|
| 100 ng–11 ng | 5-fold dilution in Adaptor Dilution Buffer |
| 10 ng–1 ng | 25-fold dilution in Adaptor Dilution Buffer |

**The NEBNext adaptor is provided in NEBNext oligos kit. NEB has several oligo kit options, which are supplied separately from the library prep kit.

2.6.2. Assemble the ligation reaction **on ice** by adding the following components, in the order given, to the end prep reaction product from Step 2.5.4:

| **LIGATION REACTION** | **VOLUME PER ONE LIBRARY** |
|---|---|
| End Prepped DNA (Step 4.5.4) | 60 µl |
| Diluted Adaptor (Step 4.6.1) | 2.5 µl |
| (red) NEBNext Ligation Enhancer | 1 µl |
| (red) NEBNext Ultra II Ligation Master Mix | 30 µl |
| **Total Volume** | **93.5 µl** |

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

2.6.3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

⚠ **Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.**

2.6.4. Incubate **15 minutes at 20°C** in a thermocycler.

2.6.5. Add 3 µl (blue) USER Enzyme to the ligation mixture from Step 4.6.4, resulting in total volume of 96.5 µl.

2.6.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C.

2.6.7. Proceed immediately to Purification of the Ligation Reaction.

### 2.7 Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

⚠ **If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A.**

2.7.1. Add 87 µl (0.9X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.7.2. Incubate for 10 minutes at room temperature.

2.7.3. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments **(Caution: do not discard the beads)**.

2.7.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.7.5. Repeat Step 4.7.4 once for a total of 2 washing steps.

2.7.6. Briefly spin the tube, and put the tube back in the magnetic rack.

2.7.7. Completely remove the residual ethanol, and air dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

2.7.8. Remove the tube from the magnet. Elute DNA target from the beads by adding 17 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times, and incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.

2.7.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

**Note: If you need to stop at this point in the protocol, samples can be stored at –20°C.**

### 2.8. PCR Enrichment of Adaptor Ligated DNA

Note: Check and verify that the concentration of your oligos is 10 µM on the label.

Use Option A for any NEBNext oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined.

2.8.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

2.8.1A **Forward and Reverse Primers Separate**

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| Adaptor Ligated DNA (Step 4.7.9) | 15 µl |
| (blue) NEBNext Ultra II Q5 Master Mix | 25 µl |
| Index (X) Primer /i7 Primer*, ** | 5 µl |
| Universal PCR Primer/i5 Primer*, ** | 5 µl |
| **Total Volume** | **50 µl** |

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

** Use only one i7 primer/ index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample

2.8.1B **Forward and Reverse Primers Combined**

| COMPONENT | VOLUME PER ONE LIBRARY |
|---|---|
| Adaptor Ligated DNA (Step 4.7.9) | 15 µl |
| (blue) NEBNext Ultra II Q5 Master Mix | 25 µl |
| Index Primer Mix* | 10 µl |
| **Total Volume** | **50 µl** |

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

2.8.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

2.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 4.8.3A and Table 4.8.3B):

**Table 2.8.3A:**

| CYCLE STEP | TEMP | TIME | CYCLES |
|---|---|---|---|
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation<br>Annealing/Extension | 98°C<br>65°C | 10 seconds<br>75 Seconds | 6–13*, ** |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ | |

*The number of PCR cycles should be adjusted based on RNA input.

**It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 7.2 in Appendix A)

**Table 2.8.3B:** Recommended PCR cycles based on input amount:

| PURIFIED mRNA or rRNA DEPLETED RNA (QUANTIFIED AFTER PURIFICATION) | RECOMMENDED PCR CYCLES |
|---|---|
| 100 ng | 6–7 |
| 50 ng | 7–8 |
| 10 ng | 9–10 |
| 1 ng | 12–13 |

**Note: PCR cycles are recommended based on high quality Universal Human Reference Total RNA. It may require optimization based on the sample quality to prevent PCR over-amplification.**

## 2.9. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

2.9.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

2.9.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

2.9.3. Incubate for 5 minutes at room temperature.

2.9.4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

**(Caution: do not discard beads)**.

2.9.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.9.6. Repeat Step 2.9.5 once for a total of 2 washing steps.

2.9.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

2.9.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down several times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

2.9.9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

**2.10. Assess Library Quality on a Bioanalyzer DNA Chip**

2.10.1. Run 1 µl library on a DNA 1000 chip. If the library yield is too low to quantify on this chip, please run the samples on a DNA High Sensitivity chip.

2.10.2. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

**Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces; Bring up the sample volume (from Step 2.9.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Beads Cleanup Step (Section 2.9).**