Appendix A

Instruments
1 fine forceps
1 fine scalpel
Insect pins
1 spoon
Coarse scissors

Other equipment
1 bottle 70% alcohol
1 bottle 90% alcohol
Gill soap
Towel, plastic sheeting
Alcohol burner + matches

Solutions
Chick Ringer’s solution
8.5g sodium chloride
0.42g potassium chloride
0.25g calcium chloride
1000 ml distilled water
100μl pen-strep antibiotic

Tyrode’s solution

Solution A
500 ml distilled water
2.0g sodium chloride
0.05 potassium chloride
0.012g N₂H₂PO₄H₂O

Solution B
480 ml distilled water
0.025g NAHCO₃

1g glucose was mixed in 20 ml distilled water in a 100 ml bottle and placed in the fridge. Solutions A and B were autoclaved separately, and the swinnexed glucose mixture was added to the solutions. 100 μl pen-strep antibiotic was added to the final solution.

**Collagenase**

0.0025g collagenase + 6 ml Tyrode’s solution

The mixture was refrigerated before use

**Culture medium**

75 % Dulbecco’s minimal essential medium (DMEM)
15 % Embryo extract
10 % Horse serum

**Retinoic acid**

All *trans* retinoic acid was dissolved in DMSO (concentration 10⁻⁵M)

**DMSO**

DMSO was dissolved in DMEM (concentration 10⁻⁵M)

**Rhodamine Phalloidin**

5μl rhodamine phalloidin + 300μl PBS

**Fibronectin**

25μl in 1ml sterile distilled water

**Solutions for protein extraction**

**Homogenization buffer, pH 7.4**

To make up 500 ml of the solution

10.00g tween 20
0.12g MgSO₄·7H₂O
0.15g CaCl₂·2H₂O
4.38g NaCl
1.51g Tris

**Solubilization buffer pH 8.0**
To make 250 ml of the solution
5.0g deoxycholate
0.3g Tris

**Tris buffer, pH 7.1**
To make 50 ml of the solution
0.01g Tris

**0.30g Mannitol 1x Running Gel Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>7%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>15.3 ml</td>
<td>12.3 ml</td>
<td>10.2 ml</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
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<tr>
<td>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</td>
<td>6.9 ml</td>
<td>9.9 ml</td>
<td>12.0 ml</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
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</tbody>
</table>

**Stacking Gel Solution (4% Acrylamide):**

<table>
<thead>
<tr>
<th>Component</th>
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<tbody>
<tr>
<td>H₂O</td>
<td>3.075 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.025 ml</td>
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<tr>
<td>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</td>
<td>0.67 ml</td>
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<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
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</table>
Silane dipped slides
Soak slides in 10% Contrad or Super 10 overnight
Rinse in hot running water—minimum—2 hours
Dry in oven at 60°C
Dip in acetone
Dip in 2% silane in acetone for 30 minutes (6 ml silane + 294 ml acetone)
Wash in two changes of acetone
Wash briefly in distilled water
Dry in 42°C incubator overnight.

Tris-HCl stock solution
100 ml of 1M Tris
76.8 ml of 1M HCl
Mix together and adjust pH to 7.6
Make up to 2 litres with distilled water

Tris Saline
42.75 g NaCl
4.5 l distilled water
Add 500 ml of Tris-HCl stock solution

Diaminobezidine (DAB)
0.01 g DAB dissolved in 2 ml of Tris-HCl stock solution. Add 20 µl freshly prepared 1% hydrogen peroxide

Phosphate Buffered Saline (PBS)
8 g NaCl
0.2 g KCl
1.15 g Na₂HPO₄
0.2 g KH₂PO₄

Hand processing schedule for embryos
Fix at least 3 hours at 4°C
Wash in phosphate buffer for 30 minutes
Place in 50% alcohol for 20 minutes
Place in 70% alcohol for 30 minutes
Place in 95% alcohol for 15 minutes
Place in 95% alcohol for 15 minutes
Place in absolute alcohol for 15 minutes
Place in absolute alcohol for 30 minutes
Place in Xylene for 10 minutes
Place in Xylene for 10 minutes
Place in wax for 15 minutes twice and for a further 30 minutes
Embed.

**Embryo extract preparation**
Glassware and solutions were sterilized the preceding day. Eleven-day-old chick eggs were cleaned with 70% alcohol, and broken into a glass dish containing a small amount of chick ringer’s solution. The head was cut off, and both the head and body were placed into another sterile glass dish, and washed. Four embryos were prepared in this manner. The prepared embryos were inserted into a 20ml syringe. Five ml was then expressed into a sterile graduated centrifuge tube. Five ml Ringer’s solution was added, and the contents were stirred with a glass rod. The contents were covered and left to stand at room temperature for one hour. Following this, the suspension was spun for 20 minutes at 2000g. After spinning, the supernatant was poured into sterile bijou bottle and frozen until use.

**India ink**
1ml in 99mls Chick Ringer’s solution

**Feulgen reaction (Feulgen and Rossenbeck, 1924)**
The paraffin sections of the chick heads or whole embryos were brought to distilled water. The sections were briefly rinsed in cold NHCL and transferred to NHCL at 60°C
for 8 minutes. As controls, similar sections of each embryo were placed in distilled water at 60°C for the same period of time. After washing in distilled water, the sections were transferred to Schiff’s reagent for 60 minutes. The sections were rinsed in three changes of sulphite rinse solution (see appendix), and then in water. The sections were counterstained in 1% aqueous light green for 1 minute, dehydrated, cleared and mounted in entellan. As a positive control, for the Feulgen-Rossenbeck method, six day old quail embryos were used.

**Titan One Tube RT-PCR system**

**Contents**

Enzyme mix  
RT-PCR buffer  
MgCl₂ stock solution  
DTT solution

**Preparation of Master mix 1**

Thaw the components listed below and place them on ice  
Vortex briefly and centrifuge all reagents before setting up the reactions  
Set up the reaction components for Master mix 1 in a separate, nuclease-free microfuge tube placed on ice.

- Sterile distilled water
- 1µl dNTP mix
- 0.4µM Downstream primer
- 0.4µM Upstream primer
- 1µG-1pg Template RNA
- 5Mm DTT Solution
5-10 U Protector RNase inhibitor

Total Volume 25μl

**Preparation of Master Mix 2**

Thaw the components listed below and place them on ice.

Vortex briefly and centrifuge all reagents before setting up the reactions.

Set up the reaction components for Master mix 2 in a separate, nuclease-free microfuge tube placed on ice.

Sterile double distilled water

1.5 mM 5X RT-PCR buffer with Mg$^{2+}$

1μL Enzyme mix
Appendix B

Statistical analysis of the distance of neural crest cells migration

At 0 degrees

<table>
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<tr>
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<th>DMEM</th>
<th>RA</th>
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<tbody>
<tr>
<td>N</td>
<td>52</td>
<td>71</td>
</tr>
<tr>
<td>Mean</td>
<td>7.84</td>
<td>3.365</td>
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<tr>
<td>Variance</td>
<td>5.6299</td>
<td>2.6782</td>
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</table>

F: 2.1021   p(same variance): 0.114

\( t: 6.9432 \)   p(same mean): 2.9359E-8

Uneq. Var. \( t \): 6.9432   p(same mean): 5.4966E-8

Permutation \( t \) test   p(same mean): 0

<table>
<thead>
<tr>
<th></th>
<th>DMEM</th>
<th>DMSO</th>
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<tbody>
<tr>
<td>N</td>
<td>52</td>
<td>59</td>
</tr>
<tr>
<td>Mean</td>
<td>7.785</td>
<td>7.84</td>
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<tr>
<td>Variance</td>
<td>5.2224</td>
<td>5.6299</td>
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</tbody>
</table>

F: 1.078   p(same variance): 0.87163

\( t: -0.074665 \)   p(same mean): 0.94087

Uneq. var. \( t \): -0.074665   p(same mean): 0.94087

Permutation \( t \) test   p(same mean): 0

One way Anova

<table>
<thead>
<tr>
<th></th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>P(same)</th>
<th>F</th>
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<tbody>
<tr>
<td>Between groups</td>
<td>263.767</td>
<td>2</td>
<td>131.884</td>
<td>1.823E-9</td>
<td>29.94</td>
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<tr>
<td>Within groups</td>
<td>257.079</td>
<td>57</td>
<td>4.51016</td>
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<tr>
<td>Total</td>
<td>520.846</td>
<td>59</td>
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### RT-PCR cycling conditions

**First strand cDNA synthesis**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Duration</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>48°C</td>
<td>45 minutes</td>
<td>reverse transcription</td>
</tr>
<tr>
<td></td>
<td>94°C</td>
<td>2 minutes</td>
<td>AMV RT inactivation and RNA/CDNA/primer denaturation</td>
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</table>

**Second strand cDNA synthesis and PCR amplification**

<table>
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<tr>
<td>40</td>
<td>94°C</td>
<td>denaturation</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>annealing</td>
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<tr>
<td></td>
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<td>extension</td>
</tr>
<tr>
<td>1</td>
<td>68°C</td>
<td>final extension</td>
</tr>
</tbody>
</table>

1 cycle 4°C soak

**Analysis**

Analyze the PCR products by agarose gel electrophoresis of 5% of the total reaction

Store the reaction products at -20°C until needed.