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 $N_2H_2PO_4H_2O$

Solution B

480 ml distilled water

0.025g $NAHCO_3$

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^{-5}M)

DMSO

DMSO was dissolved in DMEM (concentration 10^{-5}M)

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$MgSO_4.7H_2O$

0.15g $CaCl_2.2H_2O$

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 Mannitol1x Running Gel Solution

	7%	**10%**	**12%**	**15%**
H_2O	15.3 ml	12.3 ml	10.2 ml	7.2 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml
20% (w/v) SDS	0.15 ml	0.15 ml	0.15 ml	0.15 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	6.9 ml	9.9 ml	12.0 ml	15.0 ml
10% (w/v) ammonium persulfate (APS)	0.15 ml	0.15 ml	0.15 ml	0.15 ml
TEMED	0.02 ml	0.02 ml	0.02 ml	0.02 ml

Stacking Gel Solution (4% Acrylamide):

H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (APS)	0.025 ml
TEMED	0.005 ml

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 (6ml 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 to7.6

Make up to 2 litres with distilled water

Tris Saline

42.75 g NaCl

4.5l distilled water

Add 500 ml of Tris-HCl stock solution

Diaminobezidene (DAB)

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

Phosphate Buffered Saline (PBS)

8g NaCl

0.2g KCl

1.15g Na_2HPO_4

0.2g KH_2PO_4

<u>Hand processing schedule for embryos</u>

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
$MgCl_2$ 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
1.5mM	5X RT-PCR buffer with Mg^{2+}
1µL	Enzyme mix

Appendix B

Statistical analysis of the distance of neural crest cells migration

<u>At 0 degrees</u>

DMEM		**RA**	
N	52	**N**	71
Mean	7.84	**Mean**	3.365
Variance	5.6299	**Variance**	2.6782

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	

DMEM		**DMSO**	
N	52	**N**	59
Mean	7.785	**Mean**	7.84
Variance	5.2224	**Variance**	5.6299

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)	

<u>One way Anova</u>

	Sum of squares	**df**	**Mean square**	**P(same)**	**F**
Between groups	263.767	2	131.884	1.823E-9	29.94
Within groups	257.079	57	4.51016		
Total	520.846	59			

RT-PCR cycling conditions

First strand cDNA synthesis	1 cycle	48ºC for 45 minutes	reverse transcription
	1 cycle	94ºC for 2 minutes	AMV RT inactivation and RNA/CDNA/primer denaturation
Second strand cDNA synthesis and PCR amplification	40 cycles	94ºC for 30 seconds	denaturation
		60ºC for 1 minute	annealing
		68ºC for 2 minutes	extension
	1 cycle	68ºC for 7 minutes	final extension
	1 cycle	4ºC	soak

Analysis

Analyze the PCR products by agarose gel electrophoresis of 5% of the total reaction
Store the reaction products at -20ºC until needed.