2

Material and Methods

ANIMAL PROCUREMENT AND MAINTENANCE

All protocols, involving animal experimentation, were approved by the Institutional Animal Ethics Committee (IAEC) vide protocol no. 984/07/2014-2 and were performed at the Animal House Facility of the Department of Zoology, Faculty of Science. Animal handling and experiments were performed in accordance with the guidelines prescribed in 'Animal care – Appendix 3' of the Drugs and cosmetics rules, 1945.

Procurement: Adult northern house geckos *Hemidactylus flaviviridis* of both sexes with intact original tails were collected from their natural habitat. These animals were screened for parasitic infestations and the ones without the same were used for the study, while the rest were released back to their environment.

Housing: All lizards used in the study were acclimatised for at least one week in the conditions of the animal house before commencement of the experiments. They were randomized, segregated into the required experimental groups and housed in wooden cages of dimensions 15X10X18 inches, with one of the larger surfaces made of clear glass. The cages had cross-ventilation by means of three grills on three surfaces. A small shutter at the side was used to collect and replace the lizards and also to place water and food in the cages. The lizards were fed with cockroach nymphs twice a week and purified water was given daily, *ad libitum*. Ambient temperature in the animal house was 35±2°C throughout the duration of the study and a 12 hr light-dark cycle was maintained.

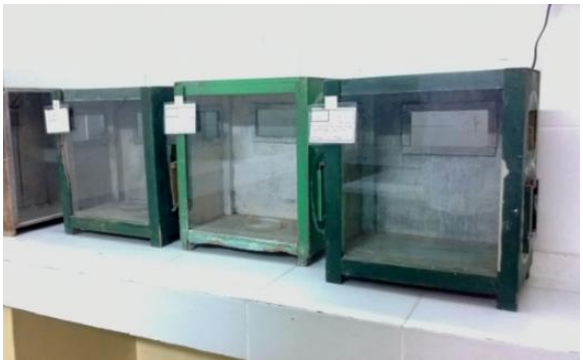

Figure 2.1: Cages used for housing lizards.

DRUG/TEST SUBSTANCE

Etoricoxib is a selective inhibitor of the inducible isoform of cyclooxygenase, *viz.*, COX-2 (*coxib* short for *cox inhibitor*). It was obtained as a gift from Sun Pharma Advanced Research Company, Vadodara in the form of a dry white powder. The compound is soluble in organic solvents and shows negligible solubility in aqueous preparations.

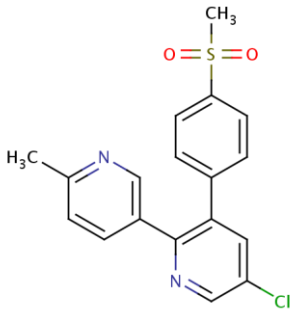

Figure 2.2: Structural formula of Etoricoxib

DRUG ADMINISTRATION

Lizards

Etoricoxib shows high absorption rate and bioavailability in man, when taken orally as tablets (Rodrigues *et al.*, 2003). Therefore the method for administration chosen for the lizards in this study was oral gavage. The dosage was decided based on a previous study by Sharma and Suresh (2008) and was set at 25 mg/kg body weight. An aqueous suspension of Etoricoxib was prepared to a concentration of 5 mg/ml and 50 µl of this suspension was administered every 24 hours till termination of the experiment. Frequency of etoricoxib administration was set at once daily based on pharmacokinetics study in humans by Agrawal et al. (2001; 2002).

Figure 2.3: Oral administration of etoricoxib to lizard

ROUTINE REAGENTS AND BUFFERS

1. Phosphate Buffered Saline (PBS)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
NaH_2PO_4	1.8 mM

pH set to 7.4

2. Tris Buffered Saline (TBS)

Tris	50 mM
NaCl	150 mM

pH set to 7.5

PROTEIN ISOLATION AND QUANTIFICATION

1. Tris-Triton Lysis Buffer

Tris base	50 mM
NaCl	200 mM
$CaCl_2$	10 mM
Triton X-100	1%

pH set to 7.5.

Protease inhibitor (Sigma, USA) was added freshly before use as per manufacturer's instructions.

2. RIPA Buffer

Tris base	50 mM
NaCl	150 mM
Triton X-100	1%
SDS	0.1%
Sodium deoxycholate	0.5%

pH set to 8.

Protease inhibitor (Sigma, USA) was added freshly before use as per manufacturer's instructions.

3. **Bradford reagent**

100 mg of Coomassie™ Brilliant Blue G250 was added to 50 ml 95% ethanol and allowed to dissolve for 2 hours. This solution was added to 100 ml 85% ortho-phosphoric acid and left overnight at room temperature. The volume was made up to 1 liter. The reagent was filtered through Whatman filter paper no. 1 and stored at 4°C in amber bottle. This reagent must have a brown-red colour.

SDS-PAGE

1. **Sample Buffer/Loading Buffer (5X)**

Tris base	250 mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.1%

pH set to 6.5.

β-Mercaptoethanol was added freshly during sample preparation at a final concentration of 100 mM.

2. **Tank Buffer**

Tris base	25 mM
Glycine	250 mM
SDS	0.2%

3. **Gel stock (30%)**

Acrylamide	29%
Bis-acrylamide	1%

Solution was kept in dark overnight at room temperature followed by filtration through Whatman paper no. 1 before use.

4. **Resolving gel buffer (12% ; 5 ml)**

30% Gel stock	2 ml
De-ionised Water	1.6 ml
1.5 M Tris Cl (pH 8.8)	1.3 ml
10% SDS	0.05 ml
10% APS	0.04 ml
TEMED	0.003 ml

APS solution was prepared fresh. APS and TEMED was added just before pouring the resolving gel buffer in the PAGE assembly.

5. **Stacking gel buffer (4% ; 3 ml)**

30% Gel stock	0.4 ml
DI water	1.8 ml
1 M Tris Cl (pH 6.8)	0.75 ml
10% SDS	0.03 ml
10% APS	0.02 ml
TEMED	0.002 ml

APS solution was prepared fresh. APS and TEMED was added just before pouring the stacking gel buffer in the PAGE assembly.

6. **Fixative for gels**

Methanol	50%
Acetic acid	10%

7. **Coommassie stain**

CBB-R250	0.1%
Methanol	40%
Acetic acid	10%

8. **Destaining solution**

Methanol	30%
Acetic acid	5%

Protocol for SDS-PAGE

Gel casting: Resolving gel buffer was poured between PAGE plates in the casting assembly, immediately followed by DI water. The gel was left undisturbed and allowed to fully polymerise for an hour. The excess water was removed and stacking gel buffer was poured on top of it. The comb was inserted immediately.

Sample preparation: Sample buffer (Loading buffer) at final working concentration, protein sample as per the required loading amount and water were added to prepare the final sample. Solution was heated at 80°C for 10 min. and placed on ice till sample was loaded onto the gel.

CBB staining: Gel was fixed in fixative for at least 20 min. and then stained in CBB staining

WESTERN BLOT

1. **Towbin Buffer for wet transfer**

Tris base	25 mM
Glycine	192 mM
Methanol	20%

pH set to 8.3. Methanol added fresh before use. Buffer made ice-cold for use.

2. **Bjerrum-Schafer-Nielsen Buffer for Semi-dry transfer**

Tris base	48 mM
Glycine	39 mM

pH set to 9.2. Buffer made ice-cold before use.

3. **Washing buffer (TBS-T)**

0.1% Triton X-100 in TBS

4. **Blocking buffer (TBS-MT)**

5% skimmed milk powder in TBS-T

5. **Anitbody dilution buffer**

BSA and Sodium azide added to TBS-T to a final concentration of 2% and 0.02% respectively.

6. **BCIP-NBT stock solution (50X)**

BCIP	9.4 mg/ml
NBT	18.75 mg/ml

Prepared in 67% DMSO solution. Diluted to working concentration in ALP substrate buffer fresh before use.

7. **ALP substrate buffer**

Tris base	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M

pH set to 9.5.

Western blot protocol

Wet transfer: After PAGE, the gel was equilibrated in Towbin transfer buffer for 20 min. A transfer stack was then prepared with the gel and membrane sandwiched between two

Whatman filters papers (no. 1) and a sponge pad of the same size on either side. Transfer was carried out in transfer unit at 100 V for 90 min.

Semi-dry transfer: Gel was equilibrated in Bjerrum-Schafer-Nielsen Buffer for 20 min. Transfer stack was prepared with gel and membrane sandwiched between four whatman papers of same size on either side. Transfer was carried out at 100 mA as per the following conditions:

Protein size range	**Transfer time**
Below 40 kDa	10 min.
40 to 70 kDa	20 min.
Above 70 kDa	25 min.

Membrane was stained in Ponceau stain to check the quality of transfer. The reversible stain was washed off with DI water and the membrane processed for antibody probing.

Antibody probing: Membrane was blocked with blocking buffer at room temperature for 1 hr. It was then incubated in primary antibody at 4°C overnight. The membrane was washed with washing buffer thrice for 10 min. each time. This was followed by incubation with biotinylated secondary antibody for 30 min. Membrane was washed again in washing buffer thrice as in the previous step, followed by incubation in Streptavidin-ALP conjugate for 15 min., and was washed again as in the previous steps. This was followed by colour development in ALP substrate.

RNA ISOLATION

1. **DEPC water**

   Prepare 0.1% DEPC in DI water and autoclave. All reagents were made in DEPC water to protect the RNA from RNase action.

2. **TBE Buffer (10X ; 1 l)**

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

   Volume made to 1 liter with DEPC water

3. **RNA loading dye (5X ; 1 ml)**

500 mM EDTA	8 µl
40% Formaldehyde	72 µl
Glycerol	200 µl
Formamide	30 µl
2.5% BPB	30 µl
10X TBE	400 µl

Volume made to 1 ml with DEPC water

4. **Other reagents used**

TRIzol reagent (Phenol + GITC)

Chloroform

2-Propanol

75% Ethanol prepared in DEPC water

1% Agarose in TBE buffer

25 mM Ethidium bromide

All glassware and disposables were sterile and all surfaces involved were cleaned prior to the experiment. The isolation was carried out using a clean pair of gloves.

RNA isolation protocol

Isolation: Tissue was homogenised in TRIzol reagent (1 ml per 100 mg tissue), followed by centrifugation at 8,000 g for 20 min. at 4°C. The supernatant was added to 200µl Chloroform. Tubes were allowed to stand on ice for 15 min. with occasional gentle shaking. The mixture was then centrifuged at 12,000 g for 15 min. at 4°C. The organic and aqueous phases separated with a visible white interphase. The upper layer was carefully taken into a new microcentrifuge tube (strictly without contamination from the lower layers) containing 400 µl isopropanol. This was gently mixed and refrigerated for at least 1 hr to allow precipitation. The tube was then centrifuged at 12,000 g for 15 min. at 4°C. The supernatant was discarded and the pellet, which contains RNA, was washed with 200 µl 75% ethanol and centrifuged again. After a total of two such washes, the tube was opened and left on ice for the pellet to dry (Overdrying was avoided since it can lead to trouble with dissolution of the RNA in water). After excess ethanol had dried off, the pellet was dissolved in 30 µl DEPC water.

Quantification: RNA was quantified using one of the two methods listed below. Both the methods were used during the study at separate times.

1. UV spectrophotometry: 1 µl RNA solution obtained from the above isolation step was diluted in 1 ml DEPC water and read at 260 nm and 280 nm on a UV-Vis spectrophotmeter (Toshniwal). The following formula was used to analyse the quantity of RNA:

$$RNA\ (\mu g/\mu l) = 40 \times OD_{260}$$

   A ratio of the OD_{260} to OD_{280} was calculated to check for quality of the RNA preparation. A ratio of around 2 was accepted as indicative of good purity of the RNA solution. Further, 3 µl of the RNA solution was electrophoresed on a 1% Agarose gel containing Ethidium bromide. Three distinct and sharp bands reflected good integrity of the RNA.

2. Qubit assay: RNA solution was diluted 1:40 and assayed on the Qubit 3.0 (Life technologies, USA) using its fluorimetric assay kit. The quality of RNA was verified by running on an Agarose gel as mentioned above.

cDNA SYNTHESIS

cDNA was synthesized from the isolated RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). The kit uses random hexamers to prime DNA polymerization reaction by the reverse transcriptase enzyme. 1 µg of total RNA was used from each sample for cDNA synthesis. The following program was set on the thermal cycler:

Temperature	Time
25°C	10 min.
37°C	120 min.
85°C	5 min.

The cDNA preparation was collected and stored at -20°C till further use.

PCR

1. **TBE buffer (10X ; 1 liter)**

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Volume made to 1 liter with DI water

2. **DNA loading dye (6X)**

Glycerol	30%
BPB	0.25%

3. **Other reagents used**

2X PCR master-mix (Sigma, USA)

Primer pairs for the respective genes

Nuclease-free water

2% Agarose in TBE buffer

25 mM Ethidium bromide

4. **Primer designing for PCR**

Primers for the current study were designed using the online Primer-BLAST and BLAST tools of the NCBI.

Sequences of the target mRNA in Zebrafish (*Danio rerio*) or Molly (*Poecilia spp.*) were aligned with those in Chick (*Gallus gallus*) using the BLAST tool. Regions of maximum sequence homology were identified and these regions of the mRNA were specified in the Primer-BLAST tool for designing of primers.

All primers were designed such that the amplicon size for each gene was below 200 bp, since real-time PCR was envisaged for gene expression studies. Primers with GC content close to 50%, melting temperature close to 60°C and minimal internally complementary sequences were selected and ordered. A list of the primer sequences used in this study has been appended at the end of this chapter.

PCR protocol

For each reaction, a reaction mix was prepared as follows:

Component	Volume
2X master-mix	5 µl
Forward primer (5 µM)	0.5 to 1 µl
Reverse primer (5 µM)	0.5 to 1 µl
cDNA template	1 µl
Nuclease-free water	to make up to 10 µl

PCRs were run on a BioRad T100 thermal cycler. The following program was used.

Temperature	Time	
95°C	3 min.	
95°C	10 sec	35 cycles
60°C	30 sec	
72°C	30 sec	

In cases of primers not annealing well at 60°C, annealing temperatures specific to those primers were used.

REAL TIME PCR

For the current study, all quantitative gene expression analyses were carried out by real time PCR using SYBR Green based master-mix (Takara Bio, Japan) on a LightCycler 96 machine (Roche Diagnostics, Switzerland).

Real-time PCR protocol

For each reaction mix, a mix with the following composition was prepared:

Component	Volume
2X SYBR Green master-mix	5 µl
Forward primer (5 µM)	0.5 µl
Reverse primer (5 µM)	0.5 µl
cDNA template	1 µl
Nuclease-free water	4 µl

For every sample, each gene was assayed in triplicate. The reactions were run in 96-well plates (Genaxy, USA) sealed with clear sealing films. The following program was used for amplification:

Temperature	**Time**	
95°C	100 sec	
95°C	10 sec	45 cycles
60°C	30 sec (acquisition)	
72°C	30 sec	

This was followed by a melt-curve analysis with the following program:

Temperature	**Time**
95°C	10 sec
65°C	60 sec
97°C	1 sec

Data analysis: Data was analysed using the LightCycler 96 software version 1.1. Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (18S rRNA) from those of the target gene. Mean of these normalized Cq values were plotted.

$$\Delta Cq = Cq\ of\ target\ gene - Cq\ of\ reference\ gene$$

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001). For fold change of gene expression in sample 2 as compared to that in sample 1:

$$\Delta\Delta Cq = \Delta Cq\ of\ gene\ in\ sample\ 2 - \Delta Cq\ of\ gene\ in\ sample\ 1$$

ΔΔCq values were plotted on a graph with vertical axis following a logarithmic scale.

IMMUNOHISTOCHEMISTRY

1. **1% Acid-alcohol**

HCl	1%
Ethanol	70%

2. **Blocking buffer**

   3% BSA in PBS

3. **Antibody dilution buffer**

   BSA and sodium azide added to PBS to final concentrations of 1% and 0.02% respectively.

3. **BCIP-NBT stock solution (50X)**

BCIP	9.4 mg/ml
NBT	18.75 mg/ml

   Prepared in 67% DMSO solution. Diluted to working concentration in ALP substrate buffer fresh before use.

4. **ALP substrate buffer**

Tris base	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M

   pH set to 9.5.

5. **Other reagents used**

   APES

   Acetone

   OCT Cryo-embedding medium (Sakura, USA)

Immunohistochemistry protocol

Coating of slides: Slides were immersed in 1% acid-alcohol for 30 min. to clean them. They were rinsed with running water, immersed in DI water and removed to dry. The dried slides were placed in acetone for 10 min. and moved into 2% APES solution (in acetone) and kept for 5 min. This was followed by 2 brief sequential rinses of DI water. The slides were dried and stored till their use.

Sectioning: Tissue was collected from the animals and, while fresh, embedded in the cryo-embedding medium at -20°C. Tissue sections in the required orientation were cut using a Cryostat microtome (Reichert Jung, USA). Sections of 8 to 12 μm thickness were cut and transferred onto the coated slides.

Antibody probing: Tissue sections were fixed in ice-cold Acetone for a minute. They were then washed with cold PBS thrice for 5 min. each time. The slides were placed in blocking buffer for an hour. The sections were then incubated in appropriately diluted primary antibody at 4°C overnight in a humidified chamber, to prevent drying. The following day, the sections were washed thrice with PBS, as mentioned above. This was followed by incubation in diluted secondary antibody for 2 hours at room temperature. The antibodies used herein were conjugated with ALP. After three washes, the sections were incubated in the ALP substrate for 5 min. and washed again. Regions on the section with the protein of interest were stained blue-purple.

Sections were mounted using DPX mountant and imaged on a Leica DM2500 microscope with a Leica EZ camera.

GREISS REACTION FOR NITRIC OXIDE

1. **Standards for reference**

   Sodium nitrite ($NaNO_2$) of concentrations 0, 20, 40, 60, 80 and 100 μM in PBS were used.

2. **Greiss reagent**

Sulfanilamide	0.1%
Phosphoric acid	2.5%
NED	0.01%

   This reagent was prepared fresh before use.

3. **Other reagents used**

   PBS for tissue lysis

   Zinc sulphate ($ZnSO_4$)

Greiss reaction protocol

Assay: The protocol was as described by Moshage et al. (1995). Tissue was weighed and homogenized in cold PBS. Zinc sulphate was added to a final concentration of 15 g/l and mixed well for precipitation of proteins. The homogenate was centrifuged at 10,000 g for 5 min. at 4°C. 100 µl supernatant was taken in a microtiter plate well, to which 100 µl Greiss reagent was added. Each sample was assayed in triplicate. After an incubation of 10 min., the plate was read at 540 nm.

Data analysis: The OD_{540} values obtained from standards were converted to µmol/mg tissue and were plotted against concentration values. The equation obtained for straight line was as follows:

$$OD = 0.003 \times \text{concentraion in } \mu M$$

COX ACTIVITY ASSAY

The COX-2 activity was assayed using a COX activity estimation kit (Cayman Chemical Co., USA). The kit measures the peroxidase activity of COX enzymes colorimetrically. The formation of oxidised TMPD is assayed at 590 nm. To selectively measure COX-2 activity, the assay was performed in presence of a specific COX-1 inhibitor.

LC/MS-MS

All solvents used in this experiment were of LC/MS grade.

LC-MS/MS protocol

A suspension of Etoricoxib was prepared in water. Animals were given 25 mg/kg body weight of the drug orally. After 24 hours of a single oral dose, tail tissue was collected by induced autotomy and a 10% homogenate was prepared in chloroform:methanol (2:1) ratio, according to the method of Folch et al. (1957). The homogenate was centrifuged at 10,000 g for 10 min. and 1 ml of the supernatant was collected in a round bottom flask of 5 ml capacity. The supernatant was vapourised in a rotary vaporizer at 50°C under high vacuum. The solid residues were washed once with acetonitrile, which was subsequently vapourised under the same conditions as mentioned above. The solid residue obtained after this washing step was dissolved in acetronitrile, and filtered through a nitrocellulose membrane of pore size 0.2 µm. This filtered solution in acetonitrile was used for LC-MS/MS analysis.

The conditions for LC/MS were similar to those used by Bräutigam et al. (2003). The mobile phase was acetonitrile-water (90:10). Flow rate was 0.3 ml/min., with an injection volume of 15 µl. Separation was carried out on an Eksigent Ekspert UltraLC 100 machine coupled with an ABSciex 3200 Q Trap machine (at the DBT-ILSPARE-sponsored Central Instrumentation Facility of the Faculty of Science).

Data was analysed on the Analyst Pro software (version 1.6.2).

SEQUENCES OF PRIMERS USED

Gene	Forward primer sequence	Reverse primer sequence
COX2	ACGTCTTGACATCACGATCCC	GGAGAAGGCTTCCCAGCTTTT
EP2	AGTTCAGCCAGAGCGAGAAC	AAGACCCAGGGGTCGATGAT
EP3	GACGATGGCGTGCAACCT	CATCTTCAGCATCGTTACCAGCA
EP4	CATTCCTCTGGTGGTCCGTG	GCTTGCAGGTCAGGGTTTTG
fgf2	ATCCGGGAGAAAAACGACCC	TTGGTCGTCTCGCTCCAAAC
fgf4	GCACCTGACCACAGGTATCC	GGCCCAGAGCAAAACATTGG
fgf8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
fgf10	GTGCGGAGCTACAATCACCT	TCCAATATGCTGAAGGGGCA
fgf12	ACCGCCAAGACAACGATGAT	CATTTCCAAGCAAGCCCCTC
fgf20	GATTCTGGGCGGCGATACTT	TCAGCTGTATCCCAGCACATC
fgf21	GAACTCACAACACGGCGTTC	CGAGTCCAGGTTGAAGTCCC
fgf22	ACACTCATCCATTTGGACTAAACTC	GACACAGATCCTTCCGGTGC
fgfr1	GCGTCACCAAAGTGGCTGTA	TACTCACGCAGGTTGCCTTT
fgfr2	GGGTTCAGCAAAGGTGCAAG	TTTGGGGTCAGGTATGCGAC
fgfr3	CGCCGACATCCTCATCTACC	GCTGTCTCTTGAGGGGGAAC
IFNγ	CCTGATGGCGTGAAGAAGGT	CTCCTCTGAGACTGGCTCCT
IL1b	CCTCCTCCAGCCAGAAAGTG	CTTGTAGCCCTTGATGCCCA
IL2	GAGCGCCAAAAGTGAGTGTG	CCTCCCCGATGACACTTTGG
IL6	TATCTATGAAGGCCGCTCCG	CCATTCCACCAACATTCGCC
IL10	AAGGAGACGTTCGAGAAGATGG	TGATGAAGATGTCGAACTCCCC
IL17	ACAGGAGATCCTCGTCCTCC	CCTTTAAGCCTGGTGCTGGA
IL22	AAGCGCTGAGTGCTGTAACT	CTTTTGGAGGTAGGGGGCTG
iNOS	AACATGCTCCTTGAGGTGGG	CAGCTCGGTCCTTCCACAAT
mmp14	GCGTACGAGAGGAATGATGGG	CGGTAGTACTGAGTCCCCTTG
mmp2	GTCTCCTGGCTCATGCCTTT	TTTCACCACTTGGCCCTCTC
mmp9	GCCTTCAAGGTGTGGAGTGA	ATCCCCGTGGTCAGCTTTTC
tgfβ2	AACCACAGAGCTGCCACTTG	CTTGGTGGGATGGCATTTTCG
timp2	ACTACGCCTGCATCAAGAGG	GGCGTGGACCAGTCTAACAT
TNFα	GGGTGTTCGCGTTGTGATTT	TCTCACTGCATCGGCTTTGT

Gene	Forward primer sequence	Reverse primer sequence
wnt1	AAGTCGGGAAGGAGAGGTGA	GAGCCATCTGAAACTGCCCT
wnt2	CGGCCCAGCAAAAATGATCT	AACTTGCACTCGCACTTGGT
wnt2B	CCTGTTGGCTGGCTATGTCT	TGTCAGCCACCATAAAGCCA
wnt3	GGAATTTGCAGACGCCAGAG	GCATGTGGTCGAGGATGGTT
wnt3A	TCCTTTGTGCCAGCATACCA	TGGATGCCGATCTTTACCCC
wnt4	CGCAAGGTGGGATCTACCAA	TTCTGTTACACTGGCGTCCC
wnt6	TTGGTCATGGACCCCAACAG	CCTCGCTGACGATTTCTGGT
wnt7A	CTGCGGAAGGGGATACAACA	TAACGTAGCAGCACCACAGG
wnt7B	TAGCCAAGGCAACCTAAGCC	CCACGCCGTACTTGACATCT
wnt8A	TAACAACGAGGCGGGAAGAC	GCAACTTCCAGACACCCCAT
wnt10A	CGAGGAGGCATTTCGACTCA	ACCGTGAACCATCCCTTTCC
wnt16	CTAAACAGTGACCAGTGCCG	GTCAGGGGTCAAGGACAGAAC