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## Protocols from: Evolutionary analyses of visual opsin genes in frogs and toads: diversity, duplication, and positive selection



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**We use this protocol and it's working**

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## Abstract

Protocols used to extract mRNA from frog retinas, create cDNA libraries, and amplify opsins by PCR for sequencing at the UT core facility under their standard protocols. These protocols were used to obtain the opsin sequences in the paper: Evolutionary analyses of visual opsin genes in frogs and toads: diversity, duplication, and positive selection.












## Materials

Frog retinas, RNeasy Kit, Qiashredder.










## Troubleshooting
















## RNA Extraction

- 1 Transfer sample into a  1.5 mL l collection tube.
- 2 Pipette off RNALATER.
- 3 Add  600  $\mu$ L Buffer RLT.
- 4 Add  6  $\mu$ L Beta-mercaptoethanol.
- 5 Disrupt tissue with sterile pestle.
- 6 Pipette into Qiashredder column. Spin  00:02:00 @ 8,000rpm. 2m
- 7 Remove Qiashredder column; Add cap; Spin  00:03:00 @ max speed. 3m
- 8 Add  600  $\mu$ L 70% Ethanol to new collection tube.
- 9 Transfer lysate to the collection tube; mix lysate and 70% Ethanol by pipetting.
- 10 Transfer lysate to RNeasy column (  700  $\mu$ L at a time). Spin  00:00:15 @ 9,800rpm; Discard flow through. Add rest of lysate; Spin  00:00:15 @ 9,800rpm; Discard flow through. 30s
- 11 Add  700  $\mu$ L Buffer RWI. Spin  00:00:15 @ 9,800rpm. 15s
- 12 Transfer RNeasy column to new collection tube.





- 13 Add  500  $\mu\text{L}$  Buffer RPE. Spin  00:00:15 @ 9,800rpm; Discard flow through. 15s
- 14 Add  500  $\mu\text{L}$  Buffer RPE – Spin  00:01:00 @ 9,800rpm; Discard Flow through/ Spin  00:02:00 @ 13,000rpm. 3m
- 15 Transfer RNeasy column to new collection tube.
- 16 Elute with  30  $\mu\text{L}$  RNase-Free H<sub>2</sub>O. Spin  00:01:00 @ 13,000rpm. 1m
- 17 Elute with  30  $\mu\text{L}$  RNase-Free H<sub>2</sub>O- Spin  00:01:00 @ 13,000rpm. 1m

## CDNA Synthesis

- 18 Combine mRNA and RNase-free H<sub>2</sub>O to standardize all samples to aliquots containing  0.4  $\mu\text{g}$  mRNA total in  10  $\mu\text{L}$  .
- 19 Make 2 Master mixes:
  - 19.1 Master Mix 1: add  1  $\mu\text{L}$  dNTP mix and  2  $\mu\text{L}$  dT primer per sample.
  - 19.2 Master Mix 2: add  4  $\mu\text{L}$  Buffer,  2  $\mu\text{L}$  DTT and  0.5  $\mu\text{L}$  RNAase inhibitor per sample.
- 20 Pipette  3  $\mu\text{L}$  of Master Mix 1 into each sample.
- 21 Place sample on dry bath at  65 °C for  00:05:00 . 5m
- 22  6.5  $\mu\text{L}$  Put samples on ice for  00:01:00 . 1m
- 23 Pipette  6.5  $\mu\text{L}$  of Master Mix 2 into each sample.



24 Pipette  1  $\mu\text{L}$  Superscript into each sample.

25  65 °C Incubate samples at room temp for  00:10:00 .

10m

26 Incubate samples at  42 °C for  00:50:00 .












50m

## PCR


17m 30s



27 Keep all reagents on ice at all times.

28 Make a master mix. Per sample add the following:

-  2.0  $\mu\text{L}$  10X Buffer
-  1.0  $\mu\text{L}$   50 millimolar (mM)  $\text{MgSO}_4$
-  0.5  $\mu\text{L}$  dNTP mix (  10 micromolar ( $\mu\text{M}$ ) each)
-  18.4  $\mu\text{L}$  ddH<sub>2</sub>O
-  1  $\mu\text{L}$  forward primer (  10 micromolar ( $\mu\text{M}$ ) )
-  1  $\mu\text{L}$  reverse primer (  10 micromolar ( $\mu\text{M}$ ) )
-  0.5  $\mu\text{L}$  Taq polymerase





29 Mix well by spinning.

30 Add  24  $\mu\text{L}$  of Master Mix to each PCR tube.

31 Add  1  $\mu\text{L}$  of sample for a total of  25  $\mu\text{L}$  per tube.

32 Program the thermocycler for the following program:

17m 30s

-  95 °C for  00:10:00
-  94 °C for  00:02:00
- REPEAT FOLLOWING 3 steps 35-50 times:



- 94 °C for 00:00:30
- 45-50 °C for 00:01:00 \*temperature depends on primer
- 72 °C for 00:02:00
- 72 °C for 00:02:00
- 4 °C hold