WP3 PROTOCOL AND PCR CONDITIONS

All PCRs are NESTED: a first PCR followed by a second PCR. Use the second PCR as template the DNA generated in the first PCR. There are several alternative primers pairs for each PCR. The final mixtures must be adjusted to the figures shown in table 1.

NOMENCLATURE: Nested PCRs are named with the acronym of the amplified fragment (RHOD, CYT5, CYT3), followed by 2 digits: the first corresponding to the condition of the first PCR and the second digit corresponding to the condition of the second PCR.
Example: RHOD-12, CYT5-21, CYT3-13 ...

ORDER OF PRIORITY: We have numbered the reactions in the order that render best results. Therefore, the priority order of any fragment is as follows: 11, 12, 13, 21, ... etc.

Table 1. PCR mixtures.

Components	Volume per reaction (µl)		Final Concentration
	RHOD	CYT-B	
10X Reaction Buffer	2.5	2.5	1X
dNTP mix (10mM of each dNTP)	1	1	0.4mM
Taq DNA polymerase (5u/µl)	0.125	0.125	1.25U/reaction
25mM $MgCl_2$	2.5	2.5	2.5mM
Downstream Primer	0.5	0.25	**0.5** - **0.25** ng/µl
Upstream Primer	0.5	0.25	**0.5** - **0.25** ng/µl
Water MQ	Fill to final volume of 25µl		

The optimal conditions for the amplification of the target fragments are as follows:

RHODOPSIN

RHOD 1st PCR	**1**	**2**	**3**
PRIMERS (25ng/μl)	F2B/5R (0.5ng/μl)	F2B/5R (0.5ng/μl)	30F/319R (0.5ng/μl)
Mg Cl_2 (25mM)	2,5 mM	2.5 mM	2.5 mM
dNTPs (10mM)	0.4 mM	0.4 mM	0.4 mM
Annealing Temp. (sec) A	62	60	62
Extesion Time (ºC) E	30	30	30

RHOD 2nd PCR	**1**	**2**	**3**	**4**
PRIMERS (25ng/μl)	F2W/R4N (0.5ng/μl)	F2X/R4N (0.5ng/μl)	F2W/R4N (0.5ng/μl)	F2X/R4N (0.5ng/μl)
Mg Cl_2 (25mM)	2.5 mM	2.5 mM	2.5 mM	2.5 mM
dNTPs (10mM)	0.4 mM	0.4 mM	0.4 mM	0.4 mM
Annealing Temp. (sec) A	56	56	54	54
Extesion Time (ºC) E	30	30	20	30

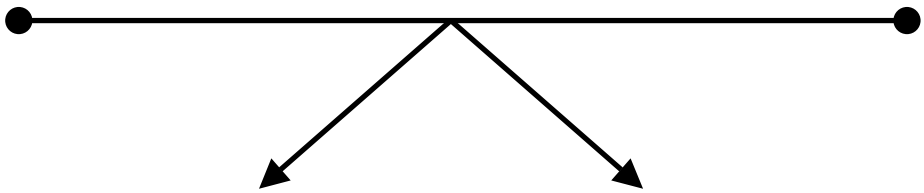
1st PCR PROGRAM:	95ºC | 94ºC | **A** | 72ºC | 72ºC | 4ºC ------- -------- ------- ------- -------- ------ 7min. 30seg. 30seg. **E** 7min. ∝
2nd PCR PROGRAM:	95ºC | 94ºC | **A** | 72ºC | 72ºC | 4ºC ------- -------- ------- ------- -------- ----- 7min. 30seg. 30seg. **E** 7min. ∝

CYTOCHROME-B

Notice that the first PCR for the Cytochrome-B is used as a template for the subsequent amplification of both Cyt-B fragments (5' and 3') in a second PCR.

Cyt-B complete (1st PCR)

CYT_B 5' and 3' 1st PCR	1	2	3	4
PRIMERS (25ng/µl)	GluFish-F / THR-Fish-R (0.25 ng/µl)	FishcytB-F/ THR-Fish-R	FishcytB-F/ TrucytB-R (0.25 ng/µl)	GluFish-F/ TrucytB-R (0.25 ng/µl)
Mg Cl_2 (25mM)	2.5 mM	2.5 mM	2.5 mM	2.5 mM
dNTPs (10mM)	0.4 mM	0.4 mM	0.4 mM	0.4 mM
Annealing Temp. (sec) A	50	50	50	50
Extesion Time (ºC) E	30	30	30	30



5' end Cyt-B (2nd PCR)

CYTB_5' 2nd PCR	1	2
PRIMERS (25ng/µl)	GluFish-F/ CytBI-5R (0.25 ng/ml)	GluFish-F/ CytBI-5R (0.25 ng/ml)
Mg Cl_2 (25mM)	2.5 mM	2.5 mM
dNTPs (10mM)	0.4 mM	0.4 mM
Annealing Temp. (sec) A	50	50
Extesion Time (ºC) E	30	30

3' end Cyt-B (2nd PCR)

CYTB_3' 2nd PCR	1	2	3
PRIMERS (25ng/µl)	TrucytB-R/ CytBI-7F (0.25 ng/ml)	TrucytB-R/ CytBI-7F (0.25 ng/ml)	TrucytB-R/ CytBI-6F (0.25 ng/ml)
Mg Cl_2 (25mM)	2.5 mM	2.5 mM	2.5 mM
dNTPs (10mM)	0.4 mM	0.4 mM	0.4 mM
Annealing Temp. (sec) A	52	50	52
Extesion Time (ºC) E	20	20	30

PCR PROGRAM:	95ºC | 94ºC | **A** | 72ºC | 72ºC | 4ºC
(1st and 2nd reactions)	7min. 30seg. 30seg. **E** 7min. ∝