2-step PCR mixture and conditions (Barcoded-head primers for seqs pooling) V.6

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protocol .

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PCR mixture and condition (2X SUPERGREEN PCR MASTER MIX)

Yin-Tse Huang 2022. 2-step PCR mixture and conditions (Barcoded-head primers for seqs pooling). **protocols.io** https://protocols.io/view/2-step-pcr-mixture-and-conditions-barcoded-head-pr-cadfsa3n Yin-Tse Huang

protocol

Herbold CW, Pelikan C, Kuzyk O, Hausmann B, Angel R, Berry D, Loy A. 2015. A flexible and economical barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. Front. Microbiol. [Internet] 6:731. Available from: http://dx.doi.org/10.3389/fmicb.2015.00731

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1 Wear glove, clean up the working bench w. 1% bleach

For 1' PCR head-primers

2 Prepare 1' PCR master mixutre for **head-primers** (**prepare 1.2X of solutions for pipetting error if needed**)

PCR mixture for head-primers for each reaction

A	B	C	D
Component	**Volume**	**Volume (1.2X)**	**Final conc.**
Forward Primer (10 µM)	1.6 µl	1.9 µl	1 µM
Reverse Primer (10 µM)	1.6 µl	1.9 µl	1 µM
2X Supergreen PCR Master Mix	7.8 µl	9.4 µl	-
ddH20	4.1 µl	4.9 µl	-
Total volume	15 µl	18 µl	-

Negative control ALWAYS NEEDED! For example, if you have 5 PCR reactions to run, prepare master mixture for 6 reactions (5 DNA template + 1 negative control).

3 Mix the 1' PCR master mixture gently by pippeting. Quick spin the tube.

4 Transfer **15 µL** 1' PCR master mixutre in 8-strip PCR tubes.

5 ⚠

Add **0.6 µL** DNA template in 8-strip PCR tubes, resulting in a **15.6 µL** reaction mixture for 1' PCR.

Negative control contains only **15 µL** master mixture but not DNA template

6 Mix the reaction mixture gently by tapping the tubes. Quick spin the tubes.

7 Carry out PCR using the following condition:

1' PCR condition for **head-primers**

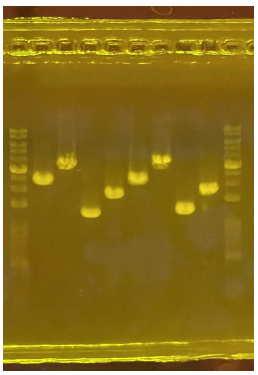
A	B	C	D
Step	**Temp**	**Sec**	**Cycle**
Initial denaturation	95 °C	30-180 (a)	
Denaturation	98 °C	15	20-25 cycles
Annealing	64-68 °C varied (b)	15	
Extension	72 °C	60-180 (c)	
Final extension	72 °C	210	
Preservation	Preservation	4 °C	∞

a. Varied depend on template complexity
b. Annealing varied, **62-65C** is working based on test on 220530; Refer to 1' PCR primers for annealing temperature
c. 1kb ~ 1min extension; enough time allow full extension of sequence

7.1 **1' hear-primers** used in Huang lab

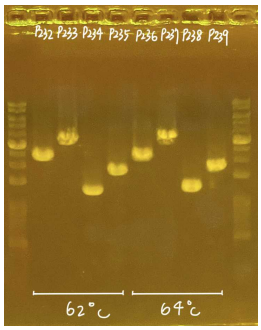
A	B	C
Name	**Tm°C**	**CG%**
NS1B1ngs_H1	71.7	60
ITS4ngs_H1	69	55.6
LR5_H1	70.2	60.6
EF1-526F_H1	71	59.3
EF1-1567R_H1	70.6	56
EF1-2218R_H1	72.2	60.3
Ben2f_H1	70.5	56.8
Bt2b_H1	74.5	62.5
T22_H1	70.3	56.8
RPB2-3bF_H1	65.6	48.7
RPB2-7cR_H1	72.3	59.7
fRPB2-11aR_H1	71.7	60.8

8 Carry out **electrophoresis** for inspection of DNA products



Gel before markdown

9 Markdown wells and upload the pictures to the Lab Google drive



Marked gel picture go to the Lab Google drive

For 2' PCR barcoded-head primers

10 Prepare 2' PCR master mixutre for **barcoded-primers** (**prepare 1.2X of solutions for pipetting error if needed**)

PCR mixture for barcoded-primers for each reaction **(NO PRIMERs at this point!!)**

A	B	C	D
Component	**Volume**	**Volume (1.2X)**	**Final conc.**
2X Supergreen PCR Master Mix	10.75 µL	12.9 µL	-
ddH20	10.75 µL	12.9 µL	-
Total volume	21.5 µL	25.8 µL	-

Negative control ALWAYS NEEDED! For example, if you have 5 PCR reactions to run, prepare master mixture for 6 reactions (5 DNA template + 1 negative control).

11 Mix the 2' PCR master mixture gently by pippeting. Quick spin the tube.

12 Transfer **21.5 µL** of the 2' PCR master mixture to PCR tubes.

13 Add **2.5 µL** **pre-mixed barcoded-head primers** (Forward + Reverse) to each PCR tube

14 ⚠

Add **1 µL** of **1' PCR product as template**, resulting in **25 µL** reaction mixture for 2' PCR.

Negative control contains only **24 µL** master mixture and premixed barcoded-head primers but not DNA template

15 Mix gently by tapping the tubes. Quick spin the tubes.

16 Carry out 2' PCR using the following condition:

2' PCR condition for **barcoded-head primers**

A	B	C	D
Step	**Temp**	**Sec**	**Cycle**
Initial denaturation	98 ºC	30	
Denaturation	98 ºC	15	10-15 cycles
Annealing	64-68 ºC varied (a)	15	
Extension	72 ºC	60 (b)	
Final extension	72 ºC	210	
Preservation	Preservation	4 ºC	∞

a. Annealing varied, **65 C** is working based on test on 220531; Refer 2' PCR primers for annealing temperature
b. 1kb ~ 1min extension; enough time allow full extension of sequence

16.1 **2' barcoded-head primers** used in Huang lab

A	B	C	D	E	F	G	H	I	J
Name (1st)	**Sequence**	**Tm°C**	**CG%**	**Name (2nd)**	**Sequence**	**Tm°C**	**CG%**	**Name (3rd)**	**Sequence**
F1-1	aaatctcattttctggtaggccggctatgcgcgagctgc	71.7	53.8	F2-1	ggccattcgtagtaagtagttcagctatgcgcgagctgc	70	53.8	F3-1	gggctagagtttaaccttaattag
F1-2	aacccgcacgtctattttaagaagctatgcgcgagctgc	70.1	51.3	F2-2	cagatactaattctcgtcacatggctatgcgcgagctgc	68.5	51.3	F3-2	taatttatggtctacagacgttag
F1-3	aactagtttatttcaaattaatagctatgcgcgagctgc	61.3	35.9	F2-3	agagattgagcttatccgtttttgctatgcgcgagctgc	68.7	48.7	F3-3	gtaagcgctcatacagcaaacta
F1-4	aatatgaagctccgacatatctggctatgcgcgagctgc	69.5	51.3	F2-4	ccattgattccagattatcatgtgctatgcgcgagctgc	67.7	48.7	F3-4	atccgcatatctaactcgaaagc
F1-5	acagtacaaacacggctcattaagctatgcgcgagctgc	70	51.3	F2-5	ccgtaggacttcgtagtttaaacgctatgcgcgagctgc	70.1	53.8	F3-5	gtgcgtgcaaatcgttttgtatcgc
F1-6	acagttgaccatccgacattatcgctatgcgcgagctgc	71.2	53.8	F2-6	gtcatgaccgctacatattctcagctatgcgcgagctgc	70	53.8	F3-6	gtggggataagcttgacattttag
F1-7	caccataaatgagattgctggaggctatgcgcgagctgc	70.7	53.8	F2-7	cgataggacaagcaatgtactcagctatgcgcgagctgc	70.1	53.8	F3-7	gttggataggaggtcaaacaga
F1-8	accgtgtatgtattcgtgttacggctatgcgcgagctgc	71	53.8	F2-8	cgatataatcgatccgccataccgctatgcgcgagctgc	71.4	56.4	F3-8	gtttaacccataagtgcgaccat
R1-1	acgtcaatgctattcccagtcaagctatgcgcgagctgc	71.4	53.8	R2-1	cagcgtttccaaagacattattggctatgcgcgagctgc	69.7	51.3	R3-1	taaaaattcctggaactccacag
R1-2	acgtcgaggtatcataaatacttgctatgcgcgagctgc	67.9	48.7	R2-2	cgctttatatgcttaaagtacccgctatgcgcgagctgc	69.1	51.3	R3-2	taatccatgtgtctattcttagagc
R1-3	cgcaagtatcgtctttcatagtcgctatgcgcgagctgc	70.1	53.8	R2-3	ctgctgagttataccacagtgacgctatgcgcgagctgc	71.5	56.4	R3-3	gtaaatctaggtgtaaaatgagt
R1-4	agtcaatgctttgggtacataaggctatgcgcgagctgc	70.1	51.3	R2-4	tacgttatattaactctagccgagctatgcgcgagctgc	67.4	48.7	R3-4	ctttcttataatatccgggctaagc
R1-5	atatcttacacaaaagtatcgttgctatgcgcgagctgc	65.4	43.6	R2-5	tagaatgtcaacacaagtaggacgctatgcgcgagctgc	69.5	51.3	R3-5	cttttgtaatggtgttgtccgttgct
R1-6	cgactattttaacttccgcaacagctatgcgcgagctgc	69.3	51.3	R2-6	gaaatcgaacaaattctgccttcgctatgcgcgagctgc	69.7	51.3	R3-6	taggtatcattctcatcctatcggc
R1-7	atcgttggttctatgttcaggtagctatgcgcgagctgc	69.5	51.3	R2-7	gaatcatcaagaagggaacaacagctatgcgcgagctgc	69.3	51.3	R3-7	tatagagacgggtttcggtaaaa
R1-8	atgagatctatctagtacccgttgctatgcgcgagctgc	68.8	51.3	R2-8	gactatagtgaaaaatcacatacgctatgcgcgagctgc	65.8	46.2	R3-8	tattagtatagatagacactcggg
R1-9	gaggagccatagagtatcaatgagctatgcgcgagctgc	69.7	53.8	R2-9	atgatcctaccggagatttacctgctatgcgcgagctgc	70.7	53.8	R3-9	tcaggggtagaagactagttgta
R1-10	atttgtagcattgaataggagcagctatgcgcgagctgc	68.7	48.7	R2-10	gagttctggatatctatgggcttgctatgcgcgagctgc	70	53.8	R3-10	tcgggttaaatgctaagcgtaatg
R1-11	caaattcctatcgtacgtgatccgctatgcgcgagctgc	70.2	53.8	R2-11	gcaacgaaacattcgttaagtatgctatgcgcgagctgc	68.1	48.7	R3-11	tgctgaaaacaggaagtctcact
R1-12	caagaactagacgctgctcttaagctatgcgcgagctgc	70.2	53.8	R2-12	tgcccaatatgttagcaccctaagctatgcgcgagctgc	71.4	53.8	R3-12	accattcctaatagccaataggg

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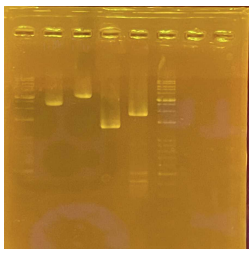

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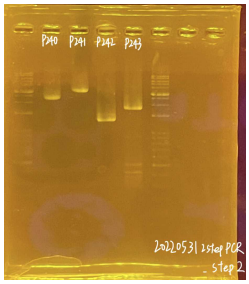

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