PROTOCOL FOR STS-PCRSEQ

Strand-and-Transcript-Specific (STS)-PCRSeq is a method to assess the efficiency of RNA editing in a discrete set of transcripts. Strand-specific primers are used to generate cDNA. The cDNA is then amplified by PCR and subjected to high-throughput sequencing. While the method has been used for assessing Arabidopsis organelle editing extent, the method could be applied to any biological system in which editing of multiple transcripts occurs.

Several steps are necessary for using STS-PCRseq to measure plant organelle editing extent

1-Extract RNA from plants.

Best method to produce high quality RNA consists in extracting RNA with Trizol then follow by using the Pure Link RNA Mini Kit (Invitrogen).

1. Grind 2-3 young leaves in liquid nitrogen, re-suspend in 800 µl of Trizol.
2. Add 160 µl of chloroform, centrifuge 13000 rpm in a microfuge for 15 minutes (cold room)
3. Recover upper phase (~400 µl)
4. Then follow the following protocol from the Pure Link RNA Mini Kit (Invitrogen).
    - 4.1. Add one volume 70% ethanol (~400 µl) to the recovered upper phase
    - 4.2. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.
    - 4.3. Transfer up to 700 µl of the sample (including any remaining precipitate) to the Spin Cartridge (with the Collection Tube).
    - 4.4. Centrifuge at 12,000 × g for 14 seconds at room temperature. Discard the flow-through, and reinsert the Spin Cartridge into the same Collection Tube.
    - 4.5. Repeat Steps 4.3–4.4 until the entire sample has been processed.
    - 4.6. Add 700 µl Wash Buffer I to the Spin Cartridge.Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through and the Collection Tube. Place the Spin Cartridge into a new Collection Tube.
    - 4.7. Add 400 µl Wash Buffer II with ethanol (page 11) to the Spin Cartridge.
    - 4.8. Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same Collection Tube.
    - 4.9. Repeat Steps 4.7–4.8 once.
    - 4.10. Centrifuge the Spin Cartridge at 12,000 × g for 1-2 minutes to dry the membrane with attached the RNA. Discard the Collection Tube and insert the Spin Cartridge into a Recovery Tube.

4.11. Add 30 µl RNase–Free Water to the center of the Spin Cartridge (see Elution Parameters, page 12).
4.12. Incubate at room temperature for 1 minute.
4.13. Centrifuge the Spin Cartridge for 2 minutes at ≥12,000 × g at room temperature to elute the RNA from the membrane into the Recovery tube.

5. DNAse the RNA with Turbo DNA free kit from Ambion
5.1. Add 3 µL (0.1 volume) 10× TURBO DNase Buffer and 1 µL TURBO DNase to the RNA, and mix gently
5.2. Incubate at 37°C for 30 min
5.3. Add 1 1 µL TURBO DNase to the RNA, and mix gently
5.4. Incubate at 37°C for 30 min
5.5..Add 3 µL resuspended DNase Inactivation Reagent and mix well
5.6. Incubate 5 min at room temperature, mixing occasionally.
5.7. Centrifuge at 10,000 × g for 1.5 min and transfer the RNA to a fresh tube

2- Reverse-transcribe organelle transcripts with strand-specific primers

Best results with the Superscript III Reverse Transcriptase (Invitrogen)

1. Mix all the plastid reverse primers in one tube (2µM) and all the mitochondrial reverse primers in another tube (2µM) (all the primers are given in the Table at the end of the protocol)

2. Perform the RT reaction with the Superscript III Reverse Transcriptase (Invitrogen)

2.1. Add the following components to a nuclease-free microcentrifuge tube:

1 µl of either plastid or mitochondrial gene-specific primer mix

1 µl total DNAsed RNA

1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)

Sterile, distilled water to 13.5 µl

2.2. Heat mixture to 65°C for 5 minutes and incubate on ice for at least 1 minute

2.3. Collect the contents of the tube by brief centrifugation and add:

4 µl 5X First-Strand Buffer

1 µl 0.1 M DTT

0.5 µl RNaseOUT™ Recombinant RNase Inhibitor (Cat. no. 10777-019,40 units/µl).

1 µl of SuperScript™ III RT (200 units/µl)*

I generally use RT diluted 20 times, which is fine to reverse transcribe organelle transcripts and saves a lot of money.

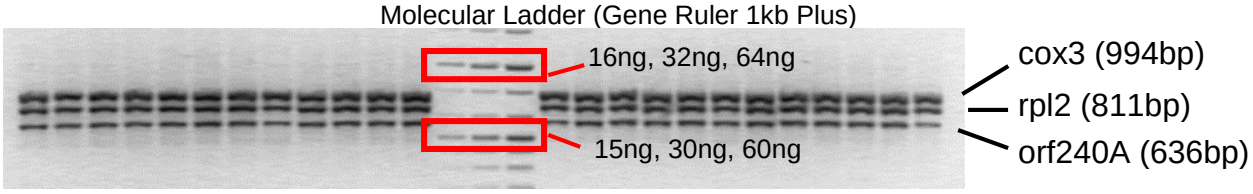
2.4. Mix by pipetting gently up and down.

2.5. Incubate at 50°C for 60 minutes.

2.6. Inactivate the reaction by heating at 70°C for 15 minutes.

3- Amplification of all mitochondrial and plastid cDNAs

Originally, all the PCR reactions were performed individually, one PCR reaction for one gene. Because there are 61 organelle genes (36 mitochondrial + 25 plastid) it means 61 PCR reactions. I recently developed a multiplex PCR strategy where I combine from 2 to 4 primer pairs, reducing the total number of PCR reactions to 25 (Primer combinations are given in a Table at the end of protocol). Significant amount of troubleshooting was necessary in order to find the appropriate primer combinations allowing amplifications to occur at roughly the same molar yield. An example is given below:



Negative of an Ethidium Bromide gel (1.5%Synergel +0.7%Agarose)

Because of the constraints on the molar yield of the amplicons, the PCR primer pairs were chosen so that the resulting PRC products sizes were in a close range (see above gel). I use the Synergel agarose additive (Diversified Biotech) that allows improved separation and definition of DNA fragments.

1. Amplification of RT-PCR products. I use the Biomix^{TM} Red (Bioline) which is is a complete ready-to-use 2x reaction mix containing an ultra-stable Taq DNA polymerase. It contains an additional inert red dye that permits easy visualization and direct loading onto a gel.

1.1. PCR mix

BioMix Red	12.5µl
Primer combination	0.1µM
cDNA	1 µl
Water (nuclease-free)	up to 25 µl

1.2. Thermal cycling conditions

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94	2 min	1
Denaturation	94	30s	45
Annealing	50	30s	
Extension	72	1 min/kb	
Final extension	72	10 min	1

2. Preparing a 1.5% Synergel + 0.7% Agrose gel

For a big tray gel, I need 300 ml of 1.5% Synergel + 0.7% Agrose or 4.5 g of Synergel and 2.1g of regular agarose

2.1. Add the Synergel™ powder to the beaker or flask and thoroughly intermix with the agarose.

2.2. Thoroughly saturate the mixed powders with ethanol. (Use enough ethanol so that the resulting slurry can just move about freely at the bottom of the container).

2.3. Add the required amount of running buffer: 300 ml of 1xTAE

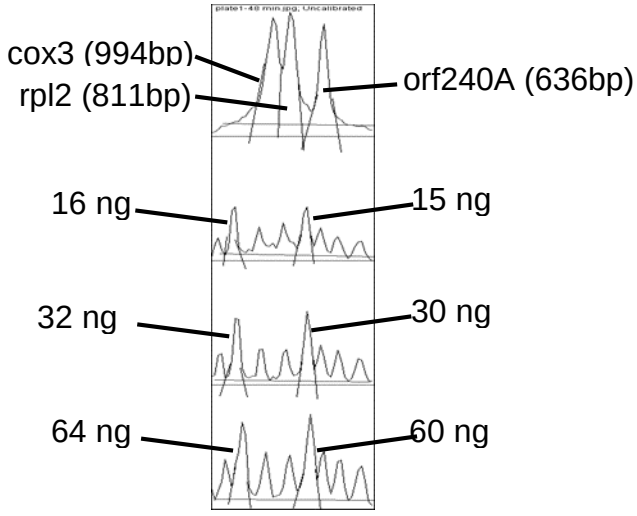
2.4. Dissolve the suspension in a microwave

2.5. Pour the gel and set the combs.

4- Quantification of the RT-PCR products

The STS-PCRseq is aimed at measuring editing extent for every organelle gene transcript and untranslated regions that have been reported to contain edited sites. In order to achieve a similar accuracy for every edited site we mix the RT-PCR products in equimolar ratio. The quantification of RT-PCR products is done by using ImageJ software on the captured image of the Ethidium Bromide loaded gel such as the one shown above (generally I load 5 µl of the PCR reaction saving 20 µl for further use in the final mix). ImageJ is a public domain Java image processing program inspired by NIH Image for the Macintosh that can calculate area and pixel value statistics of user-defined selections (http://imagej.nih.gov/ij/docs/intro.html).

As an example I am showing the resulting image from the plot lane function obtained with one of the lane from the gel above and the three molecular ladder lanes



The number of pixels under the molecular ladder peaks allows the establishment of a linear relationship between pixel intensity and amount in ng. This relationship is then used to determine the amount of the RT-PCR products.

After quantifying all the RT-PCR products, they are mixed up in equimolar ratio (100 fmoles/RT-PCR)

5- Cleaning of the RT-PCR products mix

In my most recent STS-PCRseq experiment, the volume of the RT-PCR mixes ranged from 50 µl to 200 µl. Before sharing the mix by sonication, they have to be cleaned from the primers, nucleotides, salts contained in the PCR reactions. I use the PureLink™ quick PCR purification kit (Invitrogen).

5.1. Add 4 volumes of Binding Buffer B2 with isopropanol to 1 volume of a PCR sample. Mix well.

5.2. Load. Pipet the sample into a PureLink® Spin Column in a Collection Tube. Centrifuge the column at >10,000 × g for 1 minute. Discard the flow-through.

5.3. Wash. Re-insert the column into the Collection Tube and add 650 µL Wash Buffer (W1) with ethanol. Centrifuge the column at >10,000 × g for 1 minute. Discard the flow-through and place the column in the same Collection Tube. Centrifuge the column at maximum speed for 2–3 minutes.

5.4. Elute. Place the column into a clean 1.7-mL Elution Tube (supplied with the kit). Add 50 µL nuclease free-water to the center of the column. Incubate the column at room temperature for 1 minute. Centrifuge the column at maximum speed for 2 minutes. The elution tube contains the purified PCR product. Store the purified DNA at 4°C for immediate use or at −20°C for long-term storage.

6- Quantification of the RT-PCR products mix by Qubit dsDNA HS Assay Kit (molecular probes)

The Qubit assay is very sensitive and target-specific using fluorescent dyes that bind specifically to the target molecule, thus minimizing the effect of contaminants-including DNA or RNA-on the result. It requires the assay to be read in the Qubit fluorometer.

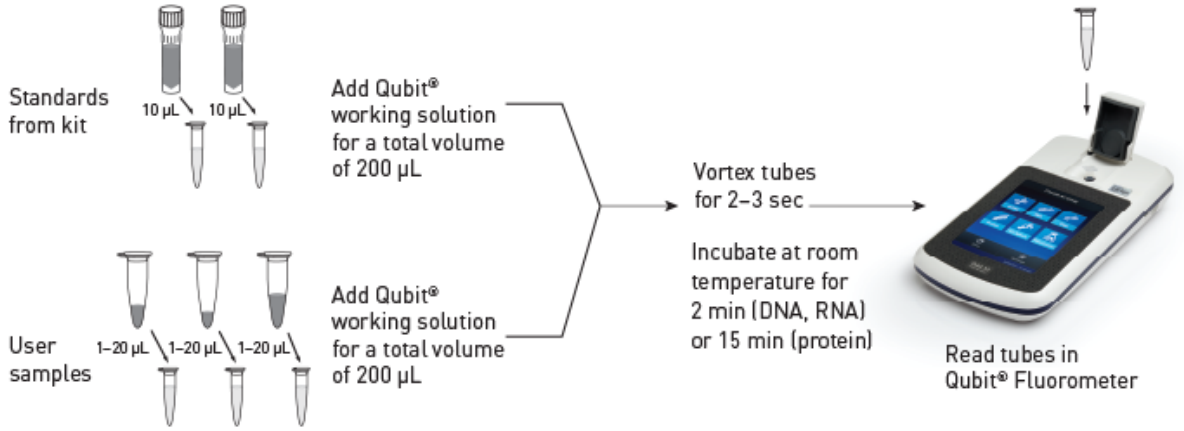
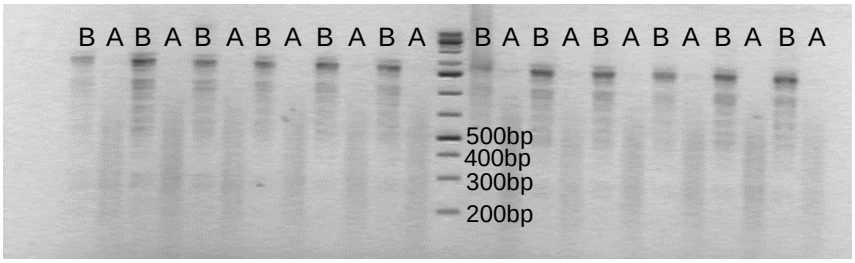


Figure from Thermofisher Scientific website

After Qubit quantification, 1200ng of each RT-PCR mix was adjusted to a sample with a volume of 60 µl and a concentration of 20ng/ µl

7- Shearing of 1.1 µg of RT-PCR mix (55 µl) by sonication with a Covaris M220 Focused Ultrasonicator

5 µl of each RT-PCR mix is set aside to monitor the efficiency of the shearing. 55 µl of the remaining RT-PCR mix is put in a Covaris microTUBE^{TM} and undergoes shearing according to the manufacturer recommendations to generate 300 bp fragments. After shearing of the mix I load a gel with neighboring lanes containing 5 µl of the mix un-sheared and 5 µl of the mix sheared



B: before shearing A: after shearing

8- Preparation of a TruSeq DNA PCR-Free library

1 µg of sheared RT-PCR mix enters the workflow of the preparation of a TruSeqDNA PCR-Free library from the HT kit (Illumina).

Citation of this protocol. The reads processing and alignment have been described in the following paper: “Comprehensive High-Resolution Analysis of the Role of an Arabidopsis Gene Family in RNA Editing”. PLOS Genetics 9: e1003584. S Bentolila S, J Oh , MR Hanson, R Bukowsky R (2013). http://www.ncbi.nlm.nih.gov/pubmed/23818871.

Primers used for organelle RT-PCR.							
purpose	organelle	gene		primers			product size (bp)
editing	plastid	*accD*	F2	ATGGAAAAATCGTGGTTCAATTTTATGTT	R2	GTTTGTCTAGTCTAATTTGAACTTCCCC	1638
editing	plastid	*atpF*	F2	TTAACCGATTCTTTCGTTTACTTG	R2	CAAACATCCCAATATTTGCATTAA	520
editing	plastid	*clp*	F2	TATTGGCGTTCCAAAAGTACCTT	R2	GAACCGCTACAAGATCAACAATTC	581
editing	plastid	*matK*	F3	ATAAATTTCAAGGATATTTAGAGTTCG	R3	TTGACCAAATCATTAAGATAAAGAATA	1501
editing	plastid	*ndhB*	F3	GCATGTACAGAATGAAAATTTCATTCTC	R2	AATCGCAATAATCGGGTTCATT	1504
editing	plastid	*ndhD*	F	AACAACTCGAAGTATGGGTC	R2	CTAATGAGAGCACAAAATCAGGAT	1637
editing	plastid	*ndhF*	F	GGATCATACCTTTCATTCCACTTC	R	GCAGCATGTATAAGAGCCGAAATG	775
editing	plastid	*ndhG*	F2	TTTGCCTGGACCAATACATG	R2	AGCCACAGAAATTGCACCTAT	517
editing	plastid	*petL*	F	AAAAAAACATATTTTATTGAGTCCCTTCATG	R	GACCAATAAACAGAACTGAGGTTATAG	101
editing	plastid	*psbE*	F2	ATGTCTGGAAGCACAGGAGA	R	CTAAAACGATCTACTAAATTCATCGAG	252
editing	plastid	*psbF*	F	GATAGGACCTATCCAATTTTTACAG	R	CGTTGGATGAACTGCATTGC	107
editing	plastid	*psbZ*	F2	TGCTTTCCAATTGGCAGTTTT	R2	TCAAGAGATAAGAGAATTAAGGATACC	181
editing	plastid	*rpl23*	F2	ATGGATGGAATCAAATATGC	R2	TTAAGTTCTTTTCTTTCTAAGAGG	282
editing	plastid	*rpoA*	F2	CTCGGACACTACAGTGGAAGTGTG	R3	TTCTACGTGAAAATGTTCAATTTTGATAAG	923
editing	plastid	*rpoB*	65F	GGTTTTATCGGTTTATTGATCAGGG	792R	CGGCGACCAATCCTTCCTAATTCAC	727
editing	plastid	*rpoB*	F2	TTCAGGTATCGACTTCAAAAGAAAC	R2	GGATACTCGGGTTCAAATACCC	472
editing	plastid	*rpoC1*	F	CCTACTTTCTTACGATTACGAGGTT	862R	ATGGGTCTCAACTCGGGAGG	374
editing	plastid	*rps12-intron*	F	ATGCCAACCATTAAACAACTT	R	TGATTGGATTTGCACCAATGGAAACC	295
editing	plastid	*rps14*	F	TTGATTTATAGGGAGAAGAAGAGGC	R	CCTGGCAACAAACATGCCTGAAC	266
splicing	plastid	*ndhA*	F1	ATGCAACAGCAGTCCAAACTATAA	R1	GAGTTGGAAAGAAGTGGTTAATAA	1061
splicing	plastid	*petB*	F1	GCATTGTATATTTCCGGAATATGA	R1	GACCGCCCAATAACCAATTT	464
splicing	plastid	*petD*	F1	CTATGAAGAGATAATGGATTATGGG	R1	AGACCTAAAGTTAGAGATTTATCAA	496
splicing	plastid	*rpl2*	F1	TGGCGATACATTTATACAAAACTT	R1	TACGGCGACGAAGAATCAAAGTCT	816
splicing	plastid	*rpl16*	F1	TCCTTTGATATAATTGCTATGCT	R1	AATAATGAATTGGGTTTTTATAGG	417
splicing	plastid	*ycf3*	F1	TCGCGTATAAATGGAAATTTTATT	R1	GAAGCGCCTCGTGATCG	492
editing	mitochondrion	*atp1*	F2	AGAGCTGCGGAACTAACGAATCTA	R5	TGATTGAGGATTCCTAATGTGATG	1537
editing	mitochondrion	*atp4 (orf25)*	F1	TGAGATTGAGTATTACGAATATGG	R1	CCCCCGAACCATTCTTA	563
editing	mitochondrion	*atp6*	F2	AATGCGACGAATCTTTTTGTTTGA	R2	CCAAGCGAACCCACTTAAAATCTT	994
editing	mitochondrion	*atp8 (orfB)*	F1	GCCTCAACTGGATAAAT	R1	TGTACAACATGGATTAGC	453
editing	mitochondrion	*atp9*	F1	ATGACAAAGCGTGAGTATAAT	R1	TCAGAATACGAATAAGATCAAA	258
editing	mitochondrion	*ccmB (ccb206)*	F3	CAGCCTTGAAGTGAATGAATT	R2	TTAATCTTGTAAACTAATCGAGACC	661
editing	mitochondrion	*ccmC (ccb256)*	F2	CTACGCGCAAATTCTCATTGG	R2	GAGCGAGTGAACTAGGTTTTGGTA	680
editing	mitochondrion	*ccmFc (ccb452)*	F2	GGTCCAACTACATAACTTTTTCTT	R2	ATTATGAACTCCACGGAACTTTCT	1350
editing	mitochondrion	*ccmFn-1 (ccb382)*	F5	ATTTCCGGGTCTTTTCGTTGCATTCACTTA	R5	CGCCGCCGAAGCAGCATGAG	1050
editing	mitochondrion	*ccmFn-2 (ccb203)*	F2	TGGACACGGGGAGGGAGCAG	R2	CATAACATAACGGGGCGGGGTTGC	601
editing	mitochondrion	*cob*	F1	GACTATAAGGAACCAACGAT	R1	ATCAGTCTCATCCGTGTAAG	1171
editing	mitochondrion	*cox2*	F2	ATGATTGTTCTAAAATGGTTATT	R1	GTTTGGGGGATTAATTGATTGG	770
editing	mitochondrion	*matR*	F3	GGGCCCTTACTCCGTCCCACAC	R2	GTCTTGACCGGGTCCGAGCTTCC	1651
editing	mitochondrion	*mttB*	F2	CACTTTTAGCTTTGAATTACTTAT	R2	ATTGATAGTTACTTTGCCAGGTTC	852
editing	mitochondrion	*nad1*	F2	CCAGCTGAAATACTTGGAATAAT	R1	AAAGGTGACTAAAAGACCAGAAAC	947
editing	mitochondrion	*nad2*	F4	GACCGTAACGTAAGTGACTCAGTG	R4	ACGGCCTACCCTTTCTTTGAA	1578
editing	mitochondrion	*nad3*	F2	AGCAAGGAGCGAGAAAACAAAGT	R2	CCCCCATTTTGTGCCCTATC	414
editing	mitochondrion	*nad4*	F4	ATGTTAGAACATTTCTGTGAATG	R1	TTTGCCATGTTGCACTAAGTTACT	1479
editing	mitochondrion	*nad4L*	F1	AATATTTCACATTTTCTATGAT	R1	AATGCTATTAATAAATTCTACAG	281
editing	mitochondrion	*nad5*	F9	ATCAGAAGGAAGCGCTATAATGACCA	R8	TAAAAACTACTCACTATCAAAATGAAAG	1917
editing	mitochondrion	*nad9*	F1	TGGATAACCAATTCATTTTCAA	R1	CGTCGCTACGCTGTTCC	567
editing	mitochondrion	*orf114*	F1	CAACCGGCGATTGGATG	R2	AATGGAAAAGGAACACCGAGTAG	381
editing	mitochondrion	*orf240A*	F1	GAGGGGAAGGTTGGTCATAAAT	R1	CGCTGGCGAAAGATACGAA	636
editing	mitochondrion	*rpl2*	F2	AGCACTTCCTCTATGGGCATTGTA	R2	TGCCTGCTTCTAATTGATGACTGG	811
editing	mitochondrion	*rpl5*	F1	ATGTTTCCACTCAATTTTCATTAC	R1	GAGTTTCCCCCTCATCTTTT	553
editing	mitochondrion	*rps3*	F1	AATCCGATTTCGGTAAGACTT	R1	CGTTTCGGATATAGCACGTC	1643
editing	mitochondrion	*rps4*	F1	TCCCCATTAAGATTTCAAACTTGTCGT	R1	TTATATGTTTTGGCCACGTCCGTTTCT	1047
editing	mitochondrion	*rps12*	F1	TCAATTGATTCGTCATGGTAGAGA	R1	TCATATCGATTTGGGTTTTTCTGC	364
editing	mitochondrion	*rps14*	F1	ATGTCGTAGAAGCAAAATAGTAGA	R1	TTATGCCCATCAAAGAACC	285
editing	mitochondrion	*nad5-1st-intron*	F1	GTGGGGCAGAGGGCTCGTAGTACC	R1	CGGTCGGGCTATCGAACACAGAGT	406
editing	mitochondrion	*nad7-2nd-intron*	F1	CTCCGCCCGGTGACTAAGAAAG	R1	AGCGTGTTCTTGGGCCATCATAG	343
editing	mitochondrion	cox3	F1	GAGGCATTCTTATCATTTG	tr-R1	TTCGGGTCATTTCTTGGTGAAC	994
editing	mitochondrion	nad6	ld-F1	AAGGGCTTGGAAGAAGAAAATG	R2	CTTTTCACCTTAGTAGTCCTATGC	653
editing	mitochondrion	nad7	ld-F1	GGAGATGCATTTCTGGTACAAGTG	R2	ATCCACCTCTCCAAACACAATA	1258
editing	mitochondrion	rps7	ld-F1	ACAAACTCGACTAAAAGAAGAGGT	R1	ATCTGAAATGCGCGAAACTT	692
editing	mitochondrion	rpl16	F1	AAAATCGATTATGCTCCTG	tr-R1	CCATACATATCGAGGGCTTTATCA	943

Primers combination for multiplex PCR

Primer combination	organelle	gene	size
1	M	nad1	947
		ccb256	680
		orf114	381
2	M	cox3	994
		rpl2	811
		orf240A	636
3	M	rps7	692
		atp4	563
		nad3	414
4	M	nad9	567
		rps12	364
		rps14	285
5	M	cob	1171
		atp6	994
		ccb206	661
6	M	rps4	1047
		nad6	653
7	M	rpl5	553
		nad5-1i	406
		nad7-2ndi	343
		atp9	258
8	M	nad7	1258
		rpl16	943
		cox2	770
9	M	rps3	1643
		nad4	1479
10	M	nad2	1578
		ccb452	1350
11	M+P	nad4L	281
		psbF	107
12	M	atp1	1537
		atp8	453
13	M	ccb382	1050
		ccb203	601
14	P	ndhD	1637
		ndhB	1504
15	P	accD	1638
		matK	1501
16	P	rpl2	816
		rpoB65	727
		ndhG	517
17	P	atpF	520
		rpl16	417
		rps14	266
18	P	clpP	581
		rpoB	472
		psbE	252
19	P	rpl23	282
		psbZ	181
		petL	101
20	P	ndhA	1061
		rpoA	923
		petD	496
21	P	ndhF	775
		ycf3	492
		rpoC1	374
22	P	petB	464
		rps12-i	295
23	M	matR	1651
24	M	nad5	1917
25	M	mttB	852