Fungal internal transcribed spacer (ITS) sequencing

Overview:

Sequencing the Yeast and fungal ITS region is useful for species identification. The ITS region has contains highly variable sequences. This is one of the most used and useful regions for species level differentiation of fungi. Attostar provides sequencing PCR master mix with primers, dNTPs, buffer and the sequencing primer. The PCR master mix amplifies the ITS region using the ITS1 and ITS4 primers (White et al, 1990).

After the PCR reaction, the sample is treated to remove primers and nucleotides, then, sent to a sequencing facility of your choice.

The sequence information may then be used at NCBI Blast for identification.

For technical support, contact Attostar@Attostar.com

Products:

Products	Catalog No.	Quantity
Attostar ITS Fungal Sequencing kit *(Store at –20C)* *Includes: 4 vials master mix, SEQITS-MM, 2 vials of Sequencing Primer, SEQITS-P, one vial of 0.5 mm glass beads SEQITS-B15. Typical use: Prepare yeast or fungal DNA, add DNA and Taq to master mix, do PCR, treat PCR product and sequence.*	**SEQITS-KIT**	**1 kit**
Fungal ITS master mix *(Store at –20C)* *Typical use: Add DNA and Taq master mix, do PCR, treat PCR product and sequence.*	**SEQITS-MM**	**0.175 ml**
ITS Sequencing Primer (Store at –20C) *Typical use: Send 10 ul to sequencing facility with PCR product.*	**SEQITS-P**	**0.1 ml**
BSA 1 mg/ml (Store at –20C) *Typical use: Add to master mix if PCR is done in LightCycler glass capillaries.*	**BSA100**	**0.1 ml**
Glass beads 0.5 mm *(2 grams in each vial)*	**SEQITS-B15**	**15 vials**

Reagents to be obtained by the user:

AmpliTaq Gold LD. Obtain from Applied Biosystems.
Catalog number 4338857(1000 units) or 438856 (250 units)
https://www2.appliedbiosystems.com/

PrepMan Ultra Sample Preparation Reagent
Catalog number 4318930
https://www2.Appliedbiosystems.com/

ExoSAP-IT
Catalog number 78200 100 reactions
http://www.usbweb.com

Sybr GreenI
Catalog number S9430 0.5 ml
http://www.sigmaaldrich.com
Typical use: Dilute a small aliquot 1:1000 in water and store at –20C.

Procedure:

Prepare Yeast or fungal DNA:

- With a 1µl loop, add yeast or fungi to a 0.2 ml tube containing 100 µl PrepMan Reagent. This should be a visibly cloudy suspension.
- Add one capful of 0.5mm glass beads (about 50 mg).
- Vortex 30 seconds.
- Heat 100C for 10 min.
- Place 2 ul of this heated solution into 0.1 ml DNase RNase free water.
- Do PCR with one of the 3 **PCR methods** described below.

Suggestion: If fungi are grown on a very thin layer of agar, it is easier to obtain a sample for sequencing, than cutting or digging in a thick agar. A thin agar layer may be made in a tube by coating the side of the tube before setting the tube aside to form the usual slant.

ExoSAP-IT treatment of PCR product:

- Mix 5 ul PCR product with 2 ul of ExoSAP-IT.
- Incubate at 37C for 15 minutes.
- Incubate at 80C for 15 minutes to inactivate ExoSAP-IT.
- Store treated PCR products at –20C if it is not shipped the same day.

Ship PCR product to sequencing facility:

- Include your PCR product and the sequencing primer SEQITS-P. A single primer is usually sufficient for an excellent result. Other sequencing primers may be used and provided on request.
- Request a FASTA report via email.
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Enter the PCR results into NCBI BLAST:

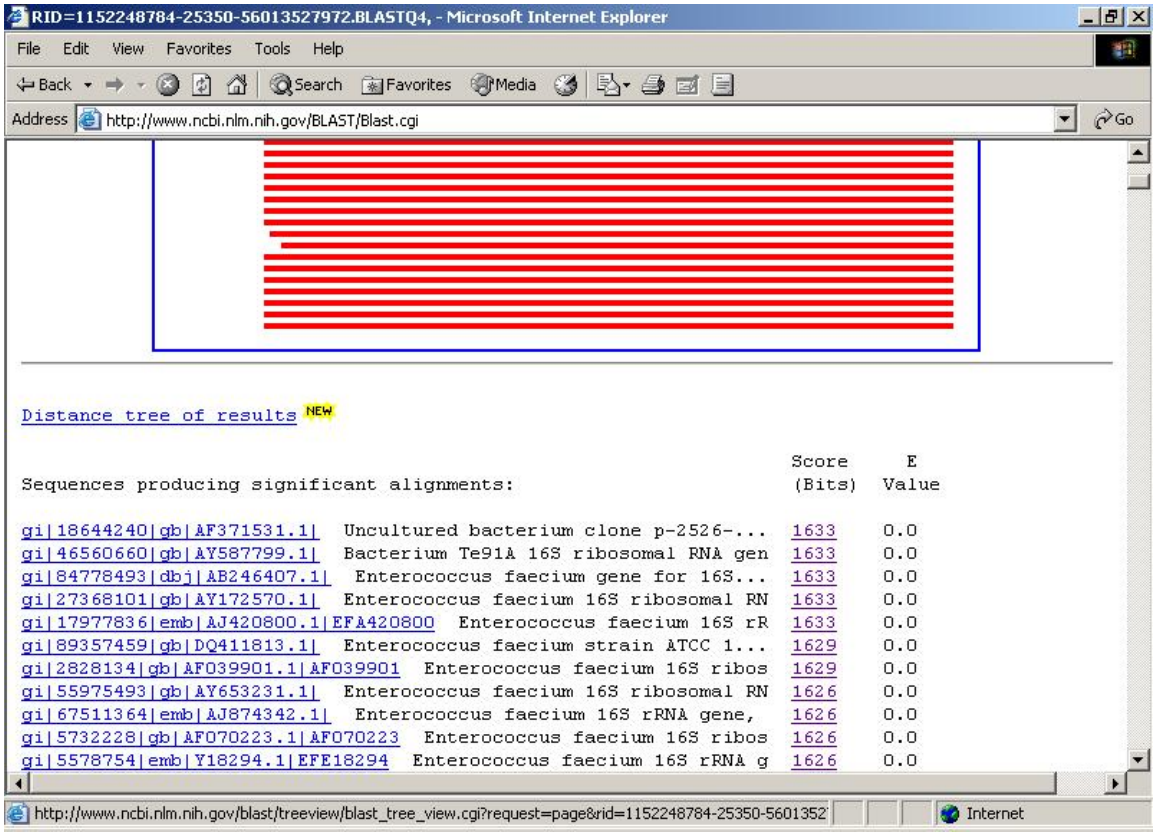
- Use this URL:

http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&LAYOUT=TwoWindows&AUTO_FORMAT=Semiauto&ALIGNMENTS=50&ALIGNMENT_VIEW=Pairwise&CLIENT=web&DATABASE=nr&DESCRIPTIONS=100&ENTREZ_QUERY=%28none%29&EXPECT=10&FILTER=L&FORMAT_OBJECT=Alignment&FORMAT_TYPE=HTML&NCBI_GI=on&PAGE=Nucleotides&PROGRAM=blastn&SERVICE=plain&SET_DEFAULTS.x=34&SET_DEFAULTS.y=8&SHOW_OVERVIEW=on&END_OF_HTTPGET=Yes&SHOW_LINKOUT=yes&GET_SEQUENCE=yes

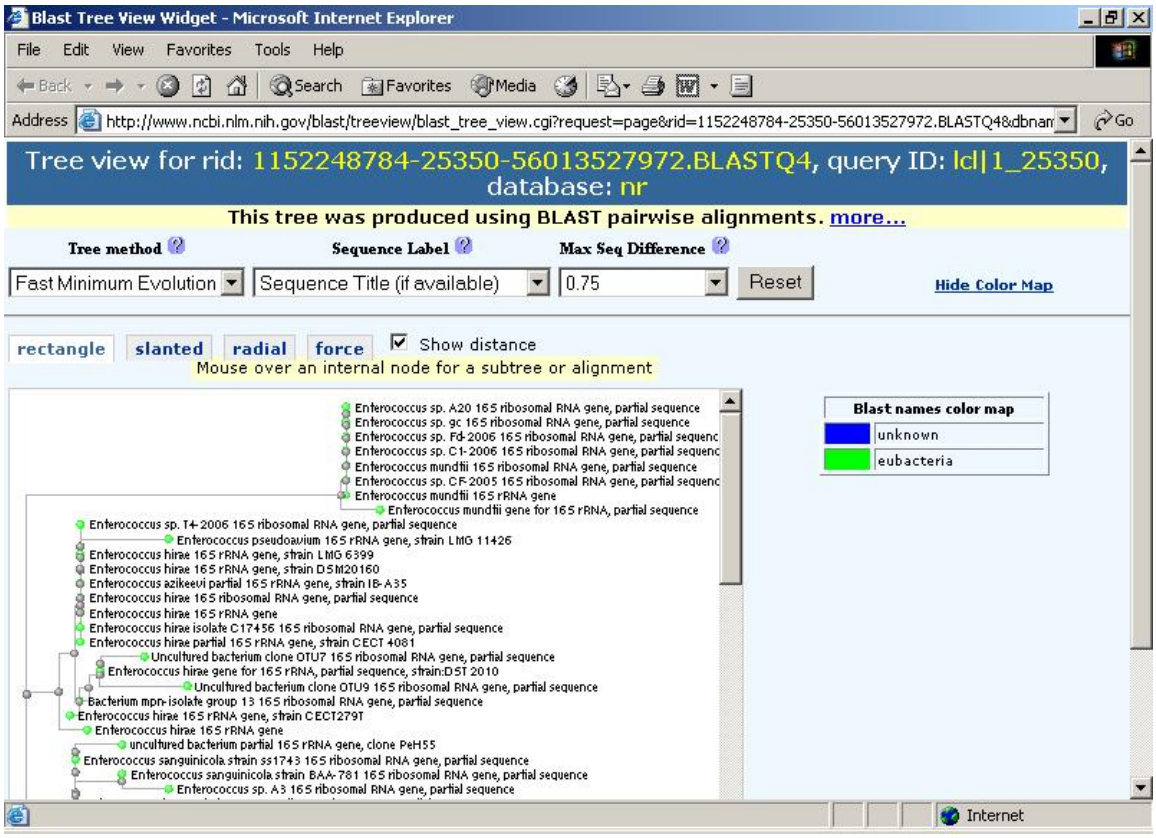
- Cut and paste the FASTA sequence results into the “enter Query sequence text box”.
- Click BLAST.
- The results will be returned very shortly.

Distance tree of results:

- Scroll down and click the “distance tree of results” for a nice display.



- You may need to elect Sequence title.
- You may need to scroll down to find your unknown



PCR methods

PCR with standard thermal cycler:

This is a robust reaction and almost always produces a good PCR product. However to be sure, gel electrophoresis of the product may be done.

Reagents needed for 24 ul final volume reactions:									
Reaction tube number	1	2	3	4	5	6	7	8	
Master mix SEQ16S-MM	19.6	39.2	58.8	78.4	98	117.6	137.2	156.8	µl
AmpliTaq Gold polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2	µl
Dispense 20 ul /tube									
Add 4 ul DNA/tube									

- Prepare master mix using the table.
- Dispense 20 microliters into 0.2 ml PCR tube.
- Add 4 ul of the bacterial DNA to the PCR tube.
- Place into thermal cycler.

Sample program using a Perkin Elmer Gene Amp 9600 thermal cycler. (or equivalent):
Thermal cycling program:

94C 10 minutes hold to activate the AmpliTaq Gold LD polymerase.
40 cycles:
53C for 60 sec
72C for 90 sec
94 for 45 sec
Final 5 min 72C hold.

- (Optional) Gel electrophoresis of PCR product.

PCR with Real-Time thermal cycler:
Adding Sybr Green to the PCR reaction permits detection of the PCR product with several real time PCR thermal cyclers. Real time detection provides evidence for production of a PCR product suitable for sequencing.

Reagents needed for 24 ul final volume reactions									
Reaction tube number	1	2	3	4	5	6	7	8	
Master mix: SEQ 16S-MM	19.6	39.2	58.8	78.4	98	117.6	137.2	156.8	µl
AmpliTaq Gold polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2	µl
SYBR Green 1:1000	1	2	3	4	5	6	7	8	µl
Dispense 21 ul / tube									
Add 3 ul DNA / tube									

- Prepare master mix using the table.
- Dispense 20 microliters into 0.2 ml PCR tube.
- Add 3 ul of the bacterial DNA to the PCR tube.
- Place into thermal cycler such as RotorGene.

Thermal cycling program:

94C 10 minutes hold to activate the AmpliTaq Gold LD polymerase.
40 cycles:
53C for 60 sec
72C for 90 sec Acquire on FAM/Sybr Green channel.
94 for 45 sec
Final 5 min 72C hold.

PCR with LightCycler using glass capillaries:
Adding Sybr Green to the PCR reaction permits detection of the PCR product with the LightCycler. LightCycler real time detection provides evidence for production of a PCR product suitable for sequencing.

LightCycler	Reagents needed for 24 ul final volume reactions								
Reaction tube number	1	2	3	4	5	6	7	8	
Master mix: SEQ 16S-MM	19.6	39.2	58.8	78.4	98	117.6	137.2	156.8	µl
AmpliTaq Gold polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2	µl
BSA 1 mg/ml	1	2	3	4	5	6	7	8	µl
SYBR Green 1:1000	1	2	3	4	5	6	7	8	µl
Dispense 22 ul / glass capillary									
Add 2 ul DNA / glass capillary									

- Prepare master mix using the table.
- Dispense 22 microliters into glass capillary.
- Add 2 ul of the bacterial DNA to the glass capillary.
- Place into LightCycler.

Thermal cycling program:
94C 10 minutes hold to activate the AmpliTaq Gold LD polymerase.
40 cycles:
53C for 60 sec
72C for 90 sec Acquire on F1 gain=1.
94 for 45 sec
72C for 5 min hold.
40C for 30 seconds cool.

- To remove from glass capillary, invert the capillary and gently centrifuge for 1 second and pipet the PCR product after removing cap.

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Reference:

White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York

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