

## Bacterial Ribosome sequencing

Bacterial Ribosome sequencing.....	1
Overview:.....	1
Products: .....	2
Attostar 16S ribosome sequencing kit   SEQ16S-KIT 1 kit .....	2
16S ribosome master mix                SEQ16S-MM 0.350 ml .....	2
Sequencing Primer                      SEQ16S-P 0.5 ml .....	2
BSA 1 mg/ml                            BSA100 0.1 ml .....	2
Reagents to be obtained by the user:.....	2
AmpliTaq Gold LD. Obtain from Applied Biosystems.....	2
PrepMan Ultra Sample Preparation Reagent .....	2
ExoSAP-IT.....	2
Sybr GreenI.....	2
PROCEDURE:.....	3
Prepare bacterial DNA:.....	3
ExoSAP-IT treatment of PCR product: .....	3
Ship PCR product to sequencing facility:.....	3
Enter the PCR results at <i>rdna.ridom.de</i> : .....	3
Enter the PCR results into Ribosomal database project.....	3
Enter the PCR results into European Ribosomal RNA database.....	3
Enter the PCR results into NCBI BLAST: .....	4
Distance tree of results:.....	4
PCR methods .....	7
PCR with standard thermal cycler: .....	7
PCR with Real-Time thermal cycler:.....	8
PCR with LightCycler using glass capillaries: .....	9

### Overview:

Sequencing 16S bacterial ribosome DNA is useful for species identification. Attostar provides a sequencing PCR master mix with primers, dNTPs, buffer and the sequencing primer. The PCR master mix amplifies a conserved region found in many bacteria. This region in the *E. coli* ribosome spans the positions 8 to 926. The exact numbering for the span in other bacteria may vary. This PCR master mix amplifies a hypervariable region, useful for species identification.

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](mailto:Attostar@Attostar.com)

**Products:**

<b>Products</b>	<b>Catalog No.</b>	<b>Quantity</b>
-----------------	--------------------	-----------------

<b>Attostar 16S ribosome sequencing kit</b>	<b>SEQ16S-KIT</b>	<b>1 kit</b>
---	-------------------	--------------

*(Store at –20C)*

*Includes: 4 vials master mix, SEQ16S-MM, 1 vials of Sequencing Primer, SEQ16S-P.*

*Typical use: Prepare bacterial DNA, add DNA and Taq to master mix, do PCR, treat PCR product and sequence.*

<b>16S ribosome master mix</b>	<b>SEQ16S-MM</b>	<b>0.350 ml</b>
--------------------------------	------------------	-----------------

*(Store at –20C)*

*Typical use: Add DNA and Taq master mix, do PCR, treat PCR product and sequence.*

<b>Sequencing Primer</b>	<b>SEQ16S-P</b>	<b>0.5 ml</b>
--------------------------	-----------------	---------------

*(Store at –20C)*

*Typical use: Send 10 ul to sequencing facility with PCR product.*

<b>BSA 1 mg/ml</b>	<b>BSA100</b>	<b>0.1 ml</b>
--------------------	---------------	---------------

*(Store at –20C)*

*Typical use: Add to master mix if PCR is done in LightCycler glass capillaries.*

**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 bacterial DNA:

- With a 1ul loop, add bacteria from an isolated colony to a 0.2 ml tube containing 50 ul PrepMan Reagent. This should be a visibly cloudy suspension.
- Vortex 10-30 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 methods described below.

### 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 SEQ16S-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.

### Enter the PCR results at [rdna.ridom.de](http://rdna.ridom.de) :

- In the “**Diagnostics**” text box, choose “Identify a microorganism by ribosomal DNA sequence”.

Or

### Enter the PCR results into Ribosomal database project

<http://rdp.cme.msu.edu/index.jsp>

- Go to Seq Match: [http://rdp.cme.msu.edu/seqmatch/seqmatch\\_intro.jsp](http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)

Or

### Enter the PCR results into European Ribosomal RNA database

<http://bioinformatics.psb.ugent.be/webtools/rRNA/>

- Go to rRNA  
BLAST: <http://bioinformatics.psb.ugent.be/webtools/rRNA/blastrrna.html>

**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](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.

Distance tree of results **NEW**

Sequences producing significant alignments:	Score (Bits)	E Value
<a href="#">gi 18644240 gb AF371531.1</a>   Uncultured bacterium clone p-2526-...	1633	0.0
<a href="#">gi 46560660 gb AY587799.1</a>   Bacterium Te91A 16S ribosomal RNA gen	1633	0.0
<a href="#">gi 84778493 dbj AB246407.1</a>   Enterococcus faecium gene for 16S...	1633	0.0
<a href="#">gi 27368101 gb AY172570.1</a>   Enterococcus faecium 16S ribosomal RN	1633	0.0
<a href="#">gi 17977836 emb AJ420800.1</a>   <a href="#">EFA420800</a> Enterococcus faecium 16S rR	1633	0.0
<a href="#">gi 89357459 gb DQ411813.1</a>   Enterococcus faecium strain ATCC 1...	1629	0.0
<a href="#">gi 2828134 gb AF039901.1</a>   <a href="#">AF039901</a> Enterococcus faecium 16S ribos	1629	0.0
<a href="#">gi 55975493 gb AY653231.1</a>   Enterococcus faecium 16S ribosomal RN	1626	0.0
<a href="#">gi 67511364 emb AJ874342.1</a>   Enterococcus faecium 16S rRNA gene,	1626	0.0
<a href="#">gi 5732228 gb AF070223.1</a>   <a href="#">AF070223</a> Enterococcus faecium 16S ribos	1626	0.0
<a href="#">gi 5578754 emb Y18294.1</a>   <a href="#">EFE18294</a> Enterococcus faecium 16S rRNA g	1626	0.0

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

Blast Tree View Widget - Microsoft Internet Explorer

File Edit View Favorites Tools Help

Address [http://www.ncbi.nlm.nih.gov/blast/treeweb/blast\\_tree\\_view.cgi?request=page&rid=1152248784-25350-56013527972.BLASTQ4&dbnr](http://www.ncbi.nlm.nih.gov/blast/treeweb/blast_tree_view.cgi?request=page&rid=1152248784-25350-56013527972.BLASTQ4&dbnr)

Tree view for rid: 1152248784-25350-56013527972.BLASTQ4, query ID: lcl|1\_25350, database: nr

This tree was produced using BLAST pairwise alignments. [more...](#)

Tree method: Fast Minimum Evolution  
 Sequence Label: Sequence Title (if available)  
 Max Seq Difference: 0.75

rectangle slanted radial force  Show distance

Mouse over an internal node for a subtree or alignment

**Blast names color map**

	unknown
	eubacteria

- Enterococcus sp. A20 16S ribosomal RNA gene, partial sequence
- Enterococcus sp. 9c 16S ribosomal RNA gene, partial sequence
- Enterococcus sp. Pd-2006 16S ribosomal RNA gene, partial sequenc
- Enterococcus sp. C1-2006 16S ribosomal RNA gene, partial sequenc
- Enterococcus mundtii 16S ribosomal RNA gene, partial sequence
- Enterococcus sp. CF-2005 16S ribosomal RNA gene, partial sequenc
- Enterococcus mundtii 16S rRNA gene
- Enterococcus mundtii gene for 16S rRNA, partial sequence
- Enterococcus sp. T4-2006 16S ribosomal RNA gene, partial sequence
- Enterococcus pseudoavium 16S rRNA gene, strain LMG 11426
- Enterococcus hirae 16S rRNA gene, strain LMG 6399
- Enterococcus hirae 16S rRNA gene, strain DSM20160
- Enterococcus azikeevi partial 16S rRNA gene, strain IB-A35
- Enterococcus hirae 16S ribosomal RNA gene, partial sequence
- Enterococcus hirae 16S rRNA gene
- Enterococcus hirae isolate C17456 16S ribosomal RNA gene, partial sequence
- Enterococcus hirae partial 16S rRNA gene, strain CECT 4081
- Uncultured bacterium clone OTU7 16S ribosomal RNA gene, partial sequence
- Enterococcus hirae gene for 16S rRNA, partial sequence, strain: DST 2010
- Uncultured bacterium clone OTU9 16S ribosomal RNA gene, partial sequence
- Bacterium mpn-isolate group 13 16S ribosomal RNA gene, partial sequence
- Enterococcus hirae 16S rRNA gene, strain CECT279T
- Enterococcus hirae 16S rRNA gene
- uncultured bacterium partial 16S rRNA gene, clone PEH55
- Enterococcus sanguinicola strain ss1743 16S ribosomal RNA gene, partial sequence
- Enterococcus sanguinicola strain BAA-781 16S ribosomal RNA gene, partial sequence
- Enterococcus sp. A3 16S ribosomal RNA gene, partial sequence

Internet

## 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 $\mu$ l
AmpliTaq Gold polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2 $\mu$ l
SYBR Green 1:1000	1	2	3	4	5	6	7	8 $\mu$ l
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 $\mu$ l
AmpliTaq Gold polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2 $\mu$ l
BSA 1 mg/ml	1	2	3	4	5	6	7	8 $\mu$ l
SYBR Green 1:1000	1	2	3	4	5	6	7	8 $\mu$ l
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.

For technical support, contact [Attostar@Attostar.com](mailto:Attostar@Attostar.com)

10-16-08