16S/18S rRNA PCR Library Creation

Version Number:	1.2
Production Start Date:	4/24/07
Version 1.2 Date:	10/01/08
Author(s):	Tanja Woyke, Doug Smith
Reviewed/Revised by:	Julianna Chow, Karla Ikeda, Eileen Dalin

Summary

This protocol is to (i) check the purity of DNA used for genome shotgun sequencing of single organisms and (ii) evaluate the diversity of an environmental DNA sample.

EH&S

JGI employees performing this procedure must wear a lab coat, safety glasses, and gloves at all times.

Note to JGI collaborators

This protocol is designed to fit into JGI's production sequencing flow. Collaborators preparing their own PCR libraries for sequencing at the JGI need to follow the procedure as outlined. It's crucial that you use the same vector and competent cells and strongly recommended that you clone your PCR products the same day of amplification and do not gel purify them. If you have to gel-purify your amplicons, use a dark reader (no UV!) for visualization and cutting of the band. If your PCR products differ from JGI's standard 16S/18S rRNA gene products, please confirm with our PMO and Cloning Technology Groups.
For each PCR library to be sequenced at the JGI, we require an agarose gel image of your QC-PCR (16-24 clones; see protocol), as well as the total kCFU's.

Materials & Reagents – PCR amplification and Cloning

Materials/Reagents/Equipment	**Vendor**	**Stock Number**
Disposables		
384-well PCR Microplate, Blue	Abgene	TF-0384/B
Clear Adhesive Plate Sealers	Edge BioSystems	48461
Reagents		
Milli-Q Water	Millipore Milli-Q System	-
27F primer (AGAGTTTGATCCTGGCTCAG)	IDT or Operon or your favorite	-

4aF primer (TCCGGTTGATCCTGCCRG)	IDT or Operon or your favorite	-
1391R primer (GACGGGCRGTGWGTRCA)	IDT or Operon or your favorite	-
515F (GTGCCAAGCAGCCGCGGTAA)	IDT or Operon or your favorite	-
1209R (GGGCATCACAGACCTG)	IDT or Operon or your favorite	-
Escherichia coli K-12 genomic DNA	ATCC	10798D-5
Archaeoglobus fulgidis genomic DNA	ATCC	49558D
Saccharomyces cerevisiae genomic DNA	Isolated In house	
Easy-A High-Fidelity PCR Master Mix	Stratagene	600640
6X loading dye	See reagent/stock preparation	-
Agarose, for routine use	Sigma	A9539-250G
50X TAE Buffer	Invitrogen	24710-030
Ultra Pure Ethidium Bromide (10mg/ml)	Invitrogen	15585011
1 Kb DNA ladder	Invitrogen	15615-016
TOPO TA Cloning Kit for Sequencing	Invitrogen	K4580-40
Equipment		
CLP Agarose gel box (12cm x 14cm) and two 25 teeth combs	CLP	75.1214-MT-25D

Procedure – PCR amplification and Cloning

NOTE: *All reagents/stock solutions should be prepared prior to the start of the procedure.*

NOTE: *Cloning should be performed on the same day as PCR amplification.*

1. **16S & 18S rRNA gene PCR Amplification**

1.1 Add **~100 ng** of DNA template to the bottom of a clean, well-labeled 96-well plate and per primer set required. If PCR inhibitors are present or the amount of DNA is limited, less DNA can be used.

NOTE: *There are three primer sets for rRNA gene amplification as follows:*

27F/1391R for bacterial 16S rRNA gene amplification (BAC 16S)

4aF/1391R for archaeal 16S rRNA gene amplification (ARCH 16S)

515F/1209R for eukaryotic 18S rRNA gene amplification (EUK 18S)

NOTE: *draw out which samples require amplification with which primer set and use different plates or plate sections for each primer set required.*

NOTE: *For environmental samples: leave 2 blank rows per row used!*

1.2 Add a positive control and a negative control per primer set used:
Add **~10 ng** of control DNA to the bottom of the 96-well plate:

E. coli DNA for BAC 16S, *A. fulgidis* genomic DNA for ARCH 16S, *Saccharomyces cerevisiae* genomic DNA for EUK 18S

1.3 Bring each sample volume up to **18.9 µl** with nuclease free H_2O.

1.4 Set-up the following PCR master mix for each primer combination for the amount of samples plus positive and negative controls. Keep plate on ice.

	1X	**12X**
F primer (10 µM)	1.8 µl	21.6 µl
R primer (10 µM)	1.8 µl	21.6 µl
Easy-A Master Mix	22.5 µl	270 µl
Total Volume:	**26.1 µl**	**313.2 µl**

1.5 Dispense **26.1 µl** of the PCR mix into each PCR plate well with template DNA. Keep plate on ice.

1.6 Spin down the plate.

1.7 Make a visual check to make sure all of the wells have mix in them (**45 µl**/ well).

1.8 Using the multi-channel pipettor, mix the samples by pipetting up and down gently. Be careful of your well location and of contamination.

1.9 **For environmental samples only:** using the multi-channel pipettor transfer 2 times 15 µl/ sample into the rows below.

NOTE: *You will end up with **3x 15 µl**/ environmental sample.*

1.10 Quick spin plate.

1.11 Set up a PE 9700 with the following PCR program (This is a 1.5 h protocol):

95 °C – 2 min.

95 °C – 40 sec.
55 °C – 30 sec.
72 °C – 90 sec.
} **20 cycles**

72°C – 7 min.
4 °C – ∞

1.12 **For environmental samples only:** after the PCR reaction, pool the samples back into one **45 µl** aliquot/ sample and mix the samples by pipetting up and down gently. Be careful of your well location and of contamination.

2. **Amplification QC using an Agarose Gel**

2.1 Transfer a **10 µl** aliquot per sample to a fresh PCR plate (or tubes).

2.2 Add **5 µl** of 1X loading dye per sample.

2.3 Vortex and spin quickly to collect the sample.

2.4 Load **15 µl** of sample onto 1% agarose gel containing Ethidium Bromide and save the remaining sample for cloning (on ice). Load **10 µl** of 1 Kb DNA ladder between the samples.

NOTE: *use smallest comb size for the agarose gel (i.e. 25 teeth combs for 12cm x 14cm agarose gel box).*

CAUTION! *Use proper safety precautions while handling Ethidium Bromide. This is a carcinogen/irritant.*

2.5 Run gel for ~ 40 min at 120V.

2.6 Image gel and check for proper inserts at **~1.4 kb** (BAC16S), **~1.4 kb** and/or **~2.1 kb** (ARCH16S) and **~0.7 kb** (EUK 18S). Make sure there is no non-specific amplification.

NOTE: *you should see a single discrete band (exceptions are possible; i.e. archaeal 16S rRNA genes with introns; some variations in rRNA gene size).*

If you see more than a discrete band of the expected size or smearing from your PCR, you either (i) optimize your PCR to eliminate non-specific amplification or (ii) gel-purify your fragment prior to cloning (avoid nuclease contamination and UV treatment when gel purifying!)

NOTE: *make sure your positive control is positive and your negative control is negative.*

2.7 Discard the gel in the appropriately labeled waste.

3. **Ligation of Amplified Product**

3.1 Add **1-4 µl** of fresh PCR product to the bottom of a clean, well-labeled 96-well plate (do not include negative controls).

NOTE: use PCR product volumes based on the intensity of the PCR product on the gel (i.e. use 4 µl, if very faint).

3.2 Add **1 µl** of dilute salt solution. (1:4 dilution of stock salt solution)

3.3 Bring the sample volume up to **5 µl** with nuclease free H_2O.

3.4 Add **1 µl** of **pCR4-TOPO vector**.

3.5 Mix gently and quick spin.

3.6 Incubate for 20 min at room temperature.

Materials & Reagents – Transformation

Materials/Reagents/Equipment	Vendor	Stock Number
Disposables		
Gene Pulse Cuvette 0.1 cm electrode gap	BioRad	165-2089
Falcon 14 ml Polypropylene Tube	Becton Dickinson	352059
Cryogenic Vial	Corning	430289
LB Carb 150 X-gal Plates	Teknova	L4940
Reagents		
ElectroMAX DH10B Cells	Invitrogen	18290015
SOC Medium	Teknova	S1640
SOC Medium	Invitrogen	Supplied with comp cells
Glycerol	Sigma	G-6279
Equipment		
Gene Pulser II	BioRad	-
Pulse Controller Plus	BioRad	-

Procedure – Transformation

NOTE: *All reagents/stock solutions should be prepared prior to the start of the procedure.*

1. **Equipment Settings (BioRad Pulse Controller):**
   - Low range: 200
   - High range: ∞ (not used)
   - Capacitance: 25
   - Voltage: 1.8 kV

2. **Transformation**

   2.1. Place on ice: one well-labeled Eppendorf tube and cuvette for each sample being transformed.

   2.2. Thaw ElectroMax DH10B competent cells on ice (each tube contains 100µl, enough for 2 reactions). Discard unused cells-DO NOT refreeze!

   2.3. To the appropriately well-labeled, cold Eppendorf tube; add **1 µl** of ligation product.

   2.4. Once thawed, mix competent cells by swirling with pipette tip a few times.

   2.5. Add **50 µl** eDH10B competent cells to the Eppendorf tube.

2.6. Mix by swirling the ligation and competent cells together with pipette tip a few times.

2.7. Transfer solution to the bottom groove of COLD cuvette and tap on tabletop a few times to settle solution to the bottom (must see even levels of cells on each side of the cuvette without bubbles).

2.8. Electroporate at 1.8 kV.

2.9. Transfer cell solution immediately to **950 µl** of SOC in a 14ml falcon tube at room temperature (make sure SOC is clear, i.e. no growth).

Important! *Transfer electroporation within 10 seconds to SOC. Rinse cuvette with* ***50 µl*** *of the same SOC mixture to which you just added the cells.*

2.10. Incubate within rotating wheel at 37°C for 1 hour.

2.11. After incubation, place on ice (no more than one hour) until ready to plate on agar plates.

3. **Plating with Beads:**

3.1. Before transforming, prepare one well-labeled LB/CARB150/IPTG/X-gal agar plate per library by letting them warm to 37˚C in an incubator to dry agar down and open (~ 90 min).

3.2. After 60 minute transformation incubation, make a 10% glycerol transformation stock (**143 µl** 80% glycerol + full transformation = ~ **1143 µl** glycerol transformation stock).

3.3. Cap, then mix transformation glycerol stock by inverting several times. [Can store tube at -80˚C if not plating within 10 min.]

3.4. Prepare a tube with **1.5 ml** of SOC (plating tube).

3.5. Add **10 µl** of the transformation glycerol stock to 1.5ml of SOC (plating tube).

3.6. Transfer remaining transformation glycerol stock into cryotube and store immediately at –80˚C for long term storage.

3.7. Mix tube by inverting several times (plating tube).

3.8. Place 10-15 beads onto bioassay.

3.9. Pipet entire volume of plating tube onto bioassay with beads (**~1510 µl**). Pipet stock over the entire plate.

3.10. Shake beads around on the bioassay until the stock has been evenly spread (Be Gentle!).

3.11. Place bioassay on benchtop allowing stock to soak in completely.

3.12. When bioassay is dry, pour beads off of bioassay into autoclave waste.

3.13. Incubate the bioassays agar down in 37˚C incubator for 16-18 hrs.

Materials & Reagents – QC PCR

Materials/Reagents/Equipment	Vendor	Stock Number

Disposables		
96-well PCR plate	USA Scientific	1402-9708
Reagents		
Taq DNA Polymerase	Amersham Biosciences	27-0799-63
10X PCR Buffer	Amersham Biosciences	-
10mM dNTP mix	MBI Fermentas	R0192
pUC-F primer (CTTTACACTTTATGCTTCC)	IDT or Operon or your favorite	-
pUC-R primer (GCAAGGCGATTAAGTTGG)	IDT or Operon or your favorite	-
1 Kb DNA ladder	Invitrogen	15615-016
Equipment		
GeneAmp PCR System 9700	Perkin Elmer (Applied Biosystems)	-

Procedure – QC PCR

Materials and Reagents – PCR QC

Materials/Reagents/Equipment	**Vendor**	**Stock Number**
Disposables		
96-well PCR plate	USA Scientific	1402-9708
Reagents		
Taq DNA Polymerase (10KU)	Amersham Biosciences	27-0799-63
10X PCR Buffer	Amersham Biosciences	-
10mM dNTP mix	MBI Fermentas	R0192
pUC-F primer (CTTTACACTTTATGCTTCC)	IDT or Operon or your favorite	-
pUC-R primer (GCAAGGCGATTAAGTTGG)	IDT or Operon or your favorite	-
FailSafe PCR System (Enzyme mix for high GC)	Epicentre	FS99250
(choose 2x Buffer K)	Epicentre	FSP995K
Equipment		
GeneAmp PCR System 9700	Perkin Elmer (Applied Biosystems)	-

Procedure – PCR for QC of Library Insert Size

1. **PCR QC**

1.1 Make up the following PCR master mix for each sample (x120 for a 96-well plate). Keep plate on ice.

	1X	**120X**
Nuclease free H_2O	16.85 µl	2022.00 µl
10X PCR Buffer	2.00 µl	240.00 µl
10mM dNTP (MBI)	0.40 µl	48.00 µl
pUC-F primer (10 pmol/µl)	0.28 µl	33.60 µl
pUC-R primer (10 pmol/µl)	0.28 µl	33.60 µl
Taq (Amersham)	0.28 µl	33.60 µl
Total Volume:	**20.09 µl**	**2410.8 µl**

1.2 Dispense **20µl** of the PCR mix into each well needed of the PCR plate. Keep plate on ice.

1.3 Spin down the plate.

1.4 Make a visual check to make sure all of the wells have mix in them.

1.5 Using pipette tips only, pick 16-24 colonies per library into their own well. Be careful of your well location and of contamination. Mix tips in cocktail well.

1.6 Quickly spin down plate.

1.7 Set up a PE 9700 with the following colony PCR program (This is a 3 hr 15 min protocol):

94 °C – 4 min.
94 °C – 30 sec.
55 °C – 30 sec.
68 °C – *2 min.
} **35 cycles**
4°C – Hold

(*modify with 5 sec. added on to each cycle for the 68 °C elongation step.)

1.8 Add **10µl** of 1X loading dye (must be faint so the PCR bands are not hidden) to PCR plate.

1.9 Vortex & spin down plate to collect.

1.10 Load **15µl** of sample onto 1% agarose gel containing Ethidium Bromide (Carcinogen/Irritant) and save the remaining sample for later use if gel fails. Leave a well empty between libraries for loading **5µl** of 1Kb ladder.

1.11 Run for ~ 30 min at 120V.

1.12 Image gel and check for inserts at the appropriate size depending upon primer set used. **~1.4 kb** (BAC16S), **~1.4 kb** and/or **~2.1 kb** (ARCH16S) and **~0.7 kb** (EUK 18S).

1.13 Discard the gel in the appropriately labeled waste.

Reagent/Stock Preparation

80% Glycerol Stock Solution

40ml 100% Glycerol (pipette slowly)
10ml Nuclease-free H_20
Autoclave
Store at room temperature

6X Loading Dye
75ml 100% glycerol
125ml Nuclease free H_20
0.05g Bromphenol Blue
0.05g Xylene Cyanole FF
Store at 4˚C

1X TAE Buffer

To make 20 liters:
400ml 50X TAE Buffer
19.6L Milli-Q H_2O
Store at room temperature

Troubleshooting

(1) Only the positive control is visible.

This may indicate that the wrong concentration of the sample DNA was used for the reaction. The concentration may be too low or too high. Low DNA concentration may produce poor amplification, while too much template DNA may inhibit the amplification reaction due to the presence of PCR inhibitors in the sample. Amplification inhibition will be most commonly be the case.

SUGGESTION:
Run template DNA serial dilutions as follows.

Dilutions:
1/10 dilution: **1 µl** of stock DNA to **9 µl** of nuclease free H_2O
1/100 dilution: **1 µl** of (1/10 dilution) to **9 µl** of nuclease free H_2O
1/1000 dilution: **1 µl** of (1/100 dilution) to **9 µl** of nuclease free H_2O

Use **1 µl** from the above dilutions for amplification reaction.

1 µl of each dilution for bacterial 16S rRNA gene amplification
1 µl of each dilution for archaeal 16S rRNA gene amplification
1 µl of each dilution for eukaryotic 18S rRNA gene amplification

Bring volume up to **18.9 µl** with nuclease free H_2O
Dispense **26.1 µl** of the appropriate PCR master mix for each primer combination.

NOTE: *For environmental samples, using multi-channel pipettor transfer 2 times **15 µl** / sample into rows below.*

NOTE: *When the above method still fails to amplify, the given gene of interest and thus superkingdom may not be present in the sample.*

(2) Both negative and positive controls are visible.

This indicates contamination.

SUGGESTION:
Toss all amplified products and repeat 16S/18S rRNA gene PCR amplification.

NOTE: *Be careful when dispensing samples to ensure no cross contamination.*

(3) Multiple bands of different sizes are visible.

This may indicate that the (environmental) sample contains organisms with rRNA genes of different lengths. Archaeal 16S rRNA genes as well as eukaryotic 18S rRNA genes may contain introns of varying size.
Moreover, unspecific amplification may occur.

SUGGESTIONS:
Repeat amplification, run a positive, negative control, and run 1ul of undiluted DNA. In addition to adding positive and negative controls, **1 µl** of DNA will be added as a DNA quality control. If multiple bands do not appear in the 1ul DNA sample, then the library should be cloned and handed off for sequencing.

NOTE: *This will help determine if the DNA was affected by the heating and cooling in the thermocycler, causing multiple band patterns.*

Add **1 µl** of DNA sample to a clean well
Bring volume up to **45 µl** with of nuclease free H_2O
Do not add any PCR master mix to the sample.

The rRNA Gene library PCR QC show different band sizes.

It is perfectly normal for ARCH 16S and EUK 18S libraries to have different size PCR product.

SOP Approval

DEPARTMENT	APPROVED BY	DATE
Lab Supervisor		
Research & Development		
Instrumentation		
QC		
Purchasing		
EH & S		
Informatics		
Seq Assessment & Analysis		

Dept Head of Prod Seq		

Appendix

AUDIT TRACKING

PROCEDURAL CHANGES