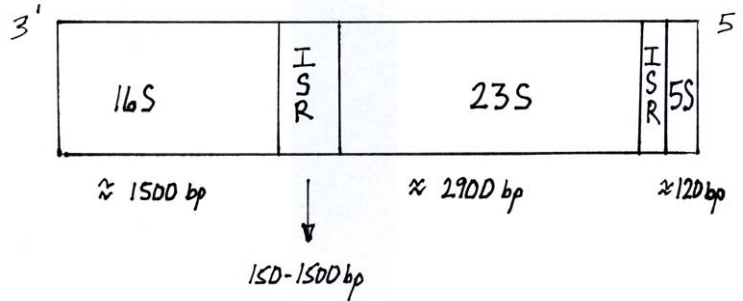


Ribosomal Intergenic Spacer analysis (RISA)

Background information

Ribosomal RNA (rRNA) intergenic spacer analysis (RISA) is a method of microbial community analysis that provides a means of comparing differing environments or treatment impacts without the bias imposed by culture-dependent approaches. RISA involves PCR amplification of a region of the rRNA gene operon between the small (16S) and large (23S) subunits called the intergenic spacer region (ISR, figure below).



By using oligonucleotide primers targeted to conserved regions in the 16S and 23S genes, RISA fragments can be generated from most of the dominant bacteria in an environmental sample. While the majority of the rRNA operon serves a structural function, portions of the 16S-23S intergenic region can encode tRNAs depending on the bacterial species. However the taxonomic value of the ISR lies in the significant heterogeneity in both length and nucleotide sequence. In RISA, we attempt to exploit the length heterogeneity of the ISR, which has been shown to range between 150 and 1500 bp with the majority of the ISR lengths being between 150 and 500 bp (figure below).

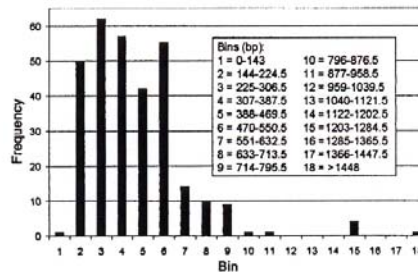
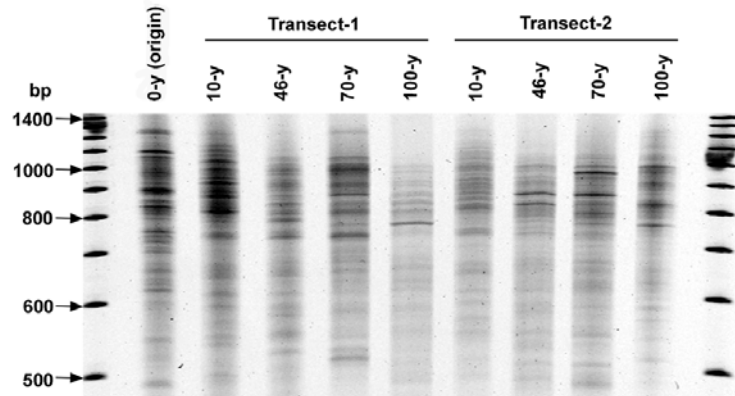


FIG. 1. Frequency histogram of 307 16S-23S intergenic spacer lengths (in base pairs) contributed by approximately 60 genera and 165 species of prokaryotes. The data were compiled from the GenBank database.

From: Fisher and Triplett. 1999. *Appl. Environ. Microbiol.* 65:4630-4636.

The resulting PCR product will be a mixture of fragments contributed by several dominant community members. This product is electrophoresed in a polyacrylamide gel, and the DNA is visualized following staining. The result is a complex banding pattern that provides a community-specific profile, with each DNA band corresponding to a bacterial population on the original assemblage (figure below).



Ribosomal intergenic spacer analysis of DNA isolated from the soils harvested along two transects of a glacier forefield. The lanes are labeled with the soil's successional age (e.g. "10-y" represents the soil harvested from ten year-old soil). The outside lanes contain DNA size markers.

Materials

PCR of the ISR

Template DNA (genomic, plasmid, bacterial colony, etc.)

Primers (resuspended in sterile water or TE to a concentration of **100 mM**)

Buffer (usually **10X**, usually sold with Taq polymerase or you can make your own)

MgCl₂ (available in 25mM or 50 mM stocks)

Bovine serum albumin (BSA, **30 mg ml⁻¹** stock)

Taq DNA polymerase

dNTPs (**2.5 mM** working solution)

Note: a 2.5 mM working solution of dNTPs means that the final concentration of each dNTP (dATP, dCTP, dGTP, and dTTP) is 2.5 mM, not that all dNTPs together make 2.5 mM. dNTPs come as 100 mM stocks. Therefore, to make the working solution thaw and add 20 µL of each dNTP to 720 µL of nuclease-free water, mix thoroughly and aliquot in 100 µl volumes. Store at -20°C.

Sterile, nuclease-free water

Gloves

PCR thermalcycler

Aerosol barrier pipet tips

PCR tubes (0.2 ml or 0.5 ml)

RISA electrophoresis

- Electrophoresis box
- Power supply
- 50 µl pipette and tips
- Pre-cast polyacrylamide gels
- Gel-star nucleic acid stain

- Wear gloves throughout the entire protocol.
- Do not cross-contaminate your samples or the solutions. Be aware of your pipette tip.
- Work clean, either on fresh blue bench paper, in the hood, or on a freshly ethanol treated bench top.

The protocol in brief

You will perform a PCR reaction using primers that amplify the 16S-23S ribosomal intergenic spacer region of most bacteria. The resulting products will be electrophoresed on pre-cast, 4% polyacrylamide gels, stained and visualized with UV transillumination.

A. RISA PCR

Review the PCR protocol and re-familiarize yourself with the materials and steps necessary to set up a PCR reaction.

Set up a PCR reaction for each of your samples as you would any other PCR reaction using the spreadsheet specific for the RISA reaction. A portion of the RISA spreadsheet is shown below. Click on the figure to access the actual RISA spreadsheet.

PCR reaction worksheet

date: 12/28/2004

desired DNA conc: 1 ng/ul

reaction size (ul) = 25 rxn # = 6

Mix	Stock conc	des conc	ul per rxn	ul all rxns	each rxn:	sample ID	mix	DNA	bulk DNA conc (ng/ul)
buffer (0 mM MgCl ₂)	10 x	1 x	2.5	15.00	1	sample 1	24.00	1.00	25
MgCl ₂	25 mM	3 mM	3.0	18.00	2	sample2	24.00	1.00	25
BSA	30 mg/ml	2 mg/ml	1.7	10.00	3	sample 3	24.00	1.00	25
each dNTP	2.5 mM	0.2 mM	2.0	12.00	4	positive control	24.00	1.00	25
primer 1406f	100 uM	0.5 uM	0.1	0.75	5	negative control	24.00	1.00	25
primer 23r	100 uM	1 uM	0.3	1.50					
H ₂ O			14.3	85.55					
taq	5 U ul ⁻¹	0.04 U ul ⁻¹	0.2	1.20					
			total	24.0					144.0
			average DNA volume:						1.00

program	cyc	temp	time
RISA	1	94 °	6'
	35	94 °30"	
		52 °30"	
		72 °1'	
	1	72 °7"	
	1	5 hold	

primers:

1	16-23S 1406f	5' - AGA GTT TGA TCC TGG CTC AG - 3'
2	16-23S 23r	5' - GGT TAC CTT GTT ACG ACT T - 3'

To set up the RISA PCR reaction, proceed as follows:

1. Wear gloves.
2. Remove the reagents and DNA from the freezer and allow them to thaw on ice. You can let them thaw at room temperature, but be sure to immediately put them back on ice once thawed.
3. Using forceps, clearly label reaction tubes (0.2 ml or 0.5 ml) as well as a master mix tube (1.5 ml).
4. Using aerosol-barrier pipette tips, add nuclease-free water to the master mix tube (95 μ l) followed by:
 - 10X PCR buffer (15 μ l)
 - MgCl₂ (9 μ l)
 - BSA (10 μ l)
 - dNTPs (12 μ l)
 - primer 1406F (5' – AGA GTT TGA TCC TGG CTC AG – 3') (0.755 μ l)
 - primer 23R (5' – GGT TAC CTT GTT ACG ACT T – 3') (1.5 μ l)
 - Taq* DNA polymerase (1.2 μ l).
5. Be sure to change the pipette tip between each reagent and not to accidentally touch the tip to any surface that might contaminate it, as the contaminating DNA could be amplified in the reaction.
6. Briefly vortex the master mix and aliquot 24 μ l into each of the five reaction tubes.
7. Add one μ l of each DNA sample to the respective tube. Remember, the negative control receives no DNA.
8. Cap the tubes, mix the contents by flicking with your finger, and then briefly (five seconds) centrifuge the tubes to concentrate the reaction mix at the bottom of the tube.
9. Place the tubes in the PCR thermalcycler, close the lid, and start the **RISA** program.

B. RISA electrophoresis

10. When the PCR is complete you will run the fragments in a polyacrylamide gel. In contrast with agarose, polyacrylamide gels provide high resolution of DNA fragments with slightly different lengths (a few bp).

11. Add an appropriate volume of loading dye (6X or 10X) to the sample (1 μ l of 6X sample dye for every 5 μ l of sample, i.e. 5 μ l of dye will be added to your 25 μ l PCR reaction).
12. Load the gel using the 50 μ l pipette. The wells of the polyacrylamide gel will hold 12 μ l, so you will load approximately half of your sample. Stick the tip below the surface of the buffer, but above the well bottom being careful not to poke a hole in the buffer well, and dispense the sample slowly. The sample will sink (due to the glycerol in the loading buffer) through the buffer and settle in the well. Load the prepared size marker.
13. After the gel has been loaded, gently place the cover on the electrophoresis box and hook up the power leads. DNA is negatively charged and will migrate toward the positive (red lead and jack in power supply) electrode. Adjust the power to between 50 and 100 volts (constant voltage). Run the gel until the first dye front (bromophenol blue) has migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) has migrated approximately one-third of the length of the gel. Do not touch the buffer when the power is on.
14. Turn off the power supply before removing the gel for staining.

C. Staining the gel

The polyacrylamide gel used for RISA analysis is stained in the same manner as the gels used in DGGE.

15. When the electrophoresis is complete, turn off the power supply, remove the gel and stain for 15 min in 50 ml of GelStar nucleic acid stain (BioWhittaker) diluted 1:10,000 in 1X TAE. Remember, GelStar binds to nucleic acids therefore it is important to minimize contact with skin, so wear gloves (powder-free). Use a container that is slightly larger than the gel. The container should be plastic and not glass.
16. Remove the gel, place it onto a UV transilluminator and view the gel.

Further reading

Borneman, J. and E.W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* 63:2647-2653.

Sigler, W.V. and J. Zeyer. 2002. Microbial diversity and activity along the forefields of two receding glaciers *Microb. Ecol.* 43:397-407.

Sigler, W.V., S. Crivii, and J. Zeyer. 2002. Bacterial succession in glacial forefield soils characterized by community structure, activity and opportunistic growth dynamics. *Microb. Ecol.* 44:306-316.