KALADRUG-R: Laboratory SOP #4 (version 147ITM107)

kDNA PCR-RFLP

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Aim

This protocol describes the procedure for comparing the identity of 2 or more DNA preparations from parasite cultures. It is based upon generation of a genetic fingerprint by PCR amplification of kDNA minicircles, followed by restriction endonuclease digestion of the obtained product and separation of the fragments according to size. The procedure is extremely sensitive to minor experimental variations, and for that reason only suitable for intra-experiment comparisons. Therefore, all DNAs that need be compared have to be run in parallel in the same experiment, and results must not be compared with fingerprints from the same DNA obtained from another experiment, even when following this same SOP.

Use: To answer the question whether parasites used in different DNA preparations generate identical patterns.

Outcome: Identical fingerprints are indicative of identical parasites.

Procedure

1. Make a list of DNA preparations that have to be compared. Identify each DNA prep with a unique identifier. Preferably, each DNA is extracted from parasite culture using the same method, even though this is probably not critical. It is advisable though to use the same solvent for each DNA, preferably TE.

2. Determine the concentration of the DNAs. This can be done using any method, *e.g.* on agarose gel or spectrophotometrically, but the same method must be used for each DNA in the same kDNA PCR-RFLP experiment.

3. Normalize the DNA concentrations to 1 ng per µl for use in PCR, with the same solvent as used to dissolve the DNA (*e.g.* TE).

4. Prepare PCR mixes as follows, in a total of 50 µl per PCR:

Component	Amount Concentration
Water	Adjust to obtain final reaction volume of 50 µl
Reaction buffer Supplied with the DNA polymerase.	1x
$MgCl_2$ This is the total amount needed. Take into account that some suppliers add	2 mM

$MgCl_2$ in the Reaction buffer	
Primer BPKDNAMINFOR 5' CTGGGGGTTGGTGTAAAATAGGGC 3'	0.5 µM
Primer BPKDNAMINREV 5' CCCGATTTTTGGCATTTTTGG 3'	0.5 µM
dGTP	200 µM
dATP	200 µM
dTTP	200 µM
dCTP	200 µM
DNA polymerase The brand is not important. The amount of units can be adjusted if necessary.	2.5 units
DNA The exact DNA amount is not critical, but should be the same for each DNA in the experiment. For this reason the concentration of each DNA must be measured with the same technology.	1 ng (1 µl)

Notes:

- The exact composition of the PCR mix is not critical, as all DNAs to be compared are run in parallel. Therefore, slight deviations of the mix, use of different enzymes, reagent batches, etc, influence all fingerprints in the same way.
- A master mix must be prepared for all PCRs in the same kDNA RFLP run, including all components except DNA. After aliquoting the master mix, the respective DNAs are added in each tube.
- Include negative control samples to check for contamination in pre-PCR and template addition areas.

5. Run the following PCR cycle:

Cycles	Time	Temperature
1	5 min	95°C
40	1 min 1 min 1 min	95°C 60°C 72°C
1	10 min	72°C

Note: If a hot start DNA polymerase is used, the initial denaturation time of 5 min may have to be increased to activate the enzyme. Follow the manufacturer's instructions.

6. Analyze 5 µl of the samples on a 2% agarose gel. Run a proper size marker along with the samples. The amplified product should be around 800 bp, and the negative controls must not show any amplicon.

7. If the PCR results are OK, proceed. If not, redo the PCR.

8. Add 5 µl 3M Na acetate pH 5.4 to the remaining 45 µl PCR product and mix.

9. Add 100 µl ice-cold absolute ethanol to each sample and mix.

10. Incubate at least 2 hours on ice or overnight at -20°C to precipitate the amplicons.

11. Spin down in a micro-centrifuge at maximum speed for 15 min.

12. Remove the supernatant without disturbing the pellet.

13. Wash the pellet with 180 µl cold 70% ethanol.

14. Spin for an additional 10 min at maximum speed and remove the supernatant.

15. Dry the pellet on air or in a vacuum centrifuge.

16. Dissolve the pellet in 10 µl water.

17. Analyze 2 µl on a 2% agarose gel using a proper size marker with known amounts of DNA.

18. Estimate the amount of each amplicon on the gel, either with the aid of software, or visually. Adjust the concentration with water to about 150 ng in 5 µl.

19. Prepare restriction digests as follows, in a total of 20 µl per reaction:

Component	**Amount Concentration**
Water	Adjust to obtain final reaction volume of 20 µl
Reaction buffer Supplied with the restriction enzyme, use conditions as specified by the manufacturer.	1x
BSA Not all manufacturers necessarily recommend BSA, others already include it in the reaction buffer.	Follow supplier
***Bsu*R1 or *Hae*III** The brand is not important, and both enzymes have the same specificity.	10 units
DNA The exact amount of amplicon used is not crucial, but should be comparable for each sample in order to visualize all fragments with the same accuracy in the subsequent gel analysis.	150 ng (5 µl)

Notes:

- A master mix must be prepared for all digests in the same kDNA RFLP run, including all components except DNA. After aliquoting the master mix, the respective DNAs are added in each tube.

20. Incubate the reactions for 2 hours at 37°C (unless otherwise specified by the manufacturer) and spin down.

21. Add 1 µl of 0.5M EDTA to stop the reaction.

22. Analyze 15 µl on a high-resolution gel, such as polyacrylamide or 3% Metaphor agarose. Load DNA samples you want to compare next to one another.

23. Compare the obtained fingerprint pattern visually. Identical parasites should result in identical patterns. If patterns are different, the parasites can be considered non-identical.

24. Make an appropriate record of your experiment, results, and conclusion.