

Restriction Enzyme Digestion

Restriction enzyme digestion is used to prepare DNA for analysis or other processing steps. Guidelines about preparing plug slices, selecting enzymes, performing DNA restriction, troubleshooting restriction steps, and using thiourea to type strains are included in this protocol.

Preparing Plug Slices

Purpose

Cut a small portion of the plug for restriction enzyme digestion and electrophoresis. Note that the size and shape of the plug slice will be reflected in the appearance of the gel pattern.

Process

Use a marked Petri dish, glass slide, or other template to cut 2 mm–thick plug slices.

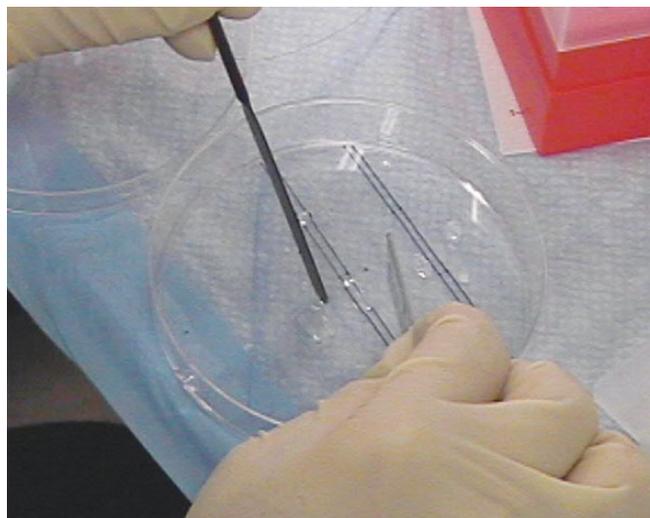
Potential Problems

- Plugs that are too small can easily be damaged and result in band distortions
- Plugs that are too large can result in thick, indistinct bands that are difficult to analyze

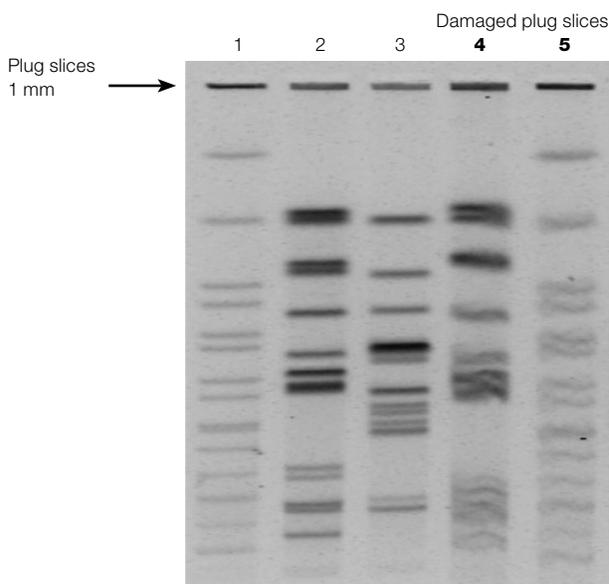
Note: Variation in plug size among samples on the same gel may result in different fluorescence intensities that can be difficult to appropriately image.

Recommendation

Only use undamaged plug slices of a consistent size.



Use a marked Petri dish, glass slide, or other template to cut 2 mm–thick plug slices.



Gel showing band distortions. Lanes 4 and 5 were loaded with small plugs that were physically damaged.

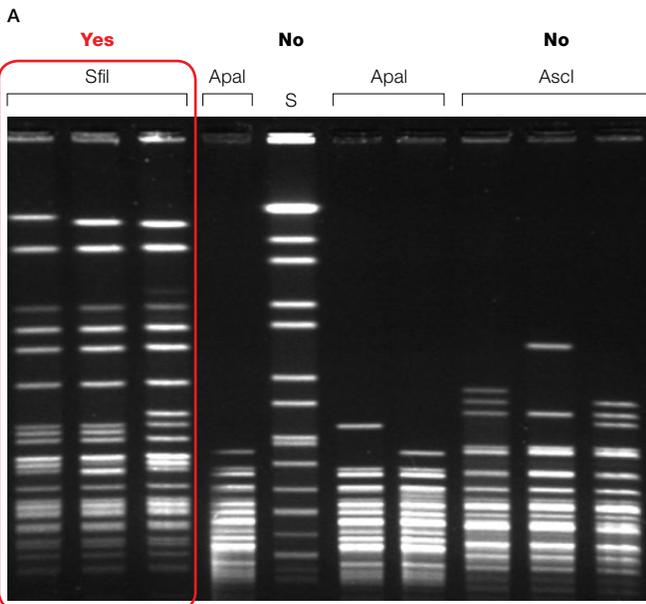
Selecting Enzymes

Purpose

The optimal restriction enzyme varies across organisms because the frequency of restriction sites varies. For pulsed field gel electrophoresis (PFGE), select a rare-cutting restriction enzyme that produces 15–20 DNA fragments across a broad range of sizes.

Process

Refer to published articles to determine what enzymes have been used previously for an organism. If the whole genome sequence of the organism is available, follow the guidelines about *in silico* DNA restriction at <http://insilico.ehu.es/>. This site identifies restriction enzymes that produce an appropriate number of DNA fragments and may be useful for PFGE analysis. The enzyme(s) should then be evaluated on a subset of isolates.



Evaluation of three restriction enzymes: Sfil, Apal, and Ascl. Sfil is the optimal enzyme for this sample. S, standard.

Performing DNA Restriction

Purpose

Use a rare-cutting restriction enzyme to cut the genomic DNA into a small number of fragments that can be resolved using PFGE.

Process

Prepare a master mix containing:

- Restriction enzyme
- Restriction buffer recommended by the enzyme vendor
- Bovine serum albumin (BSA; optional, but recommended)
- Sterile deionized water

Mix thoroughly and aliquot into separate tubes. Check the appropriate temperature for the restriction enzyme being used and incubate 2–4 hr.

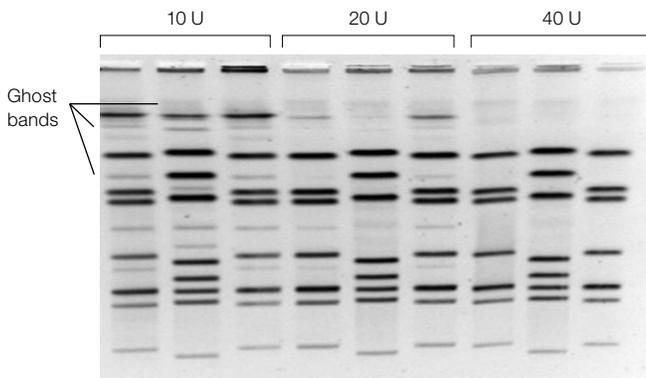
Recommendations

- Always use master mixes when preparing enzyme mixtures
- Always use the restriction buffer recommended by the vendor
- Include BSA even with enzymes that do not require it
- Routinely check water bath temperatures
- Remove enzyme master mix and incubate digested slices with 200 ml 0.5× TBE buffer to inactivate enzyme after the incubation step is complete

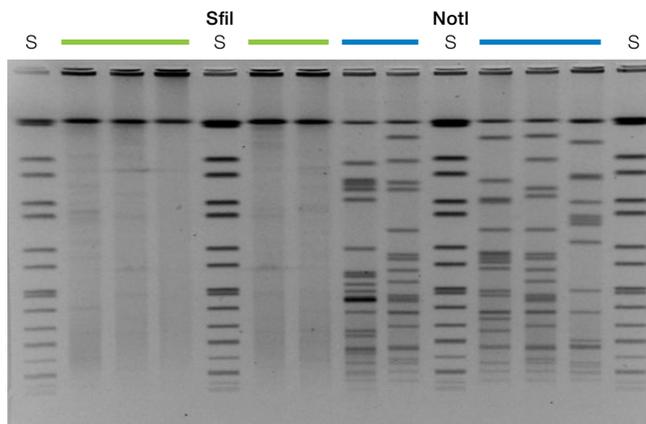
Troubleshooting Restriction Steps

Potential Problems

1. Incomplete DNA restriction appears as “shadow” or “ghost” bands on gels and can be caused by:
 - Procedure
 - Poor plug quality — proteinase K not removed from the plug
 - DNA concentration too high
 - Enzyme concentration too low
 - Bad lot of enzyme and/or buffer
 - Enzyme and/or buffer has deteriorated over time
 - Incorrect incubation temperature or buffer



Incomplete digestion due to low enzyme concentration results in ghost bands. Complete digestion was achieved by increasing the enzyme concentration to 40 U. U, units.



Lanes 2–4, 6, and 7 show smears caused by expired SfiI (—) enzyme that did not cut DNA. The same plugs cut with NotI (—) enzyme yielded distinct bands, indicating that the plugs were usable and smears were due to using expired enzyme. S, standard.

Recommendations

If incomplete restriction is observed:

- Wash the plugs 2 additional times with TE buffer
- Ensure that sufficient units of enzyme are present in the reaction by increasing the number of units of enzyme
- Make sure the DNA concentration is not too high by reducing the initial cell suspension concentration or cutting thinner slices
- Add high-quality enzyme and buffer that have been stored properly and are not expired to the master mix

Tip: To determine whether incomplete restriction is the result of enzyme or buffer quality, set up another reaction comparing the current enzyme with a new or previously tested lot of enzyme.

- BSA can be added to reaction mixtures even when not recommended by the vendor

Note: BSA helps to maintain enzyme stability during the digestion step. It also minimizes enzyme inactivation due to inhibitors and prevents the enzymes from adhering to the wall of reaction tubes.

Potential Problems

2. When exposed to nonstandard reaction conditions, some restriction enzymes manifest altered specificity and cleave sequences that are similar but not identical to their recognition sequence. This phenomenon is called “star activity” (Barany 1988). Star activity results in DNA degradation, smearing, and few bands.

Conditions that can lead to star activity include:

- Prolonged incubation time
- Incorrect buffer or buffer concentration
- High (>5% v/v) glycerol concentration
- High enzyme to μg of DNA ratio (that is, too much enzyme)

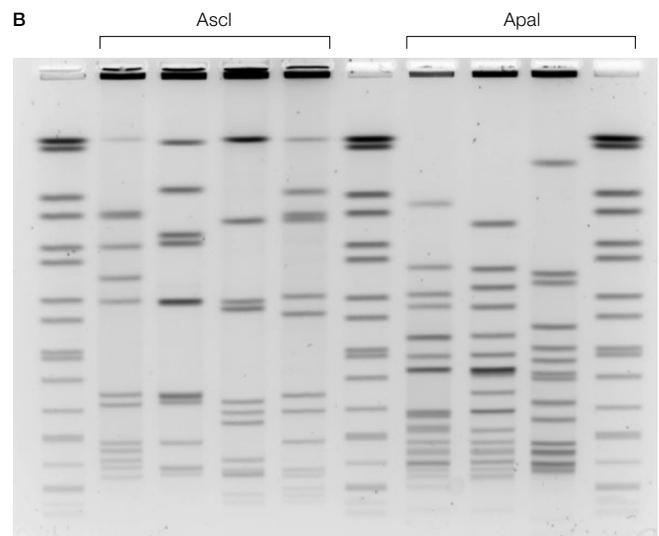
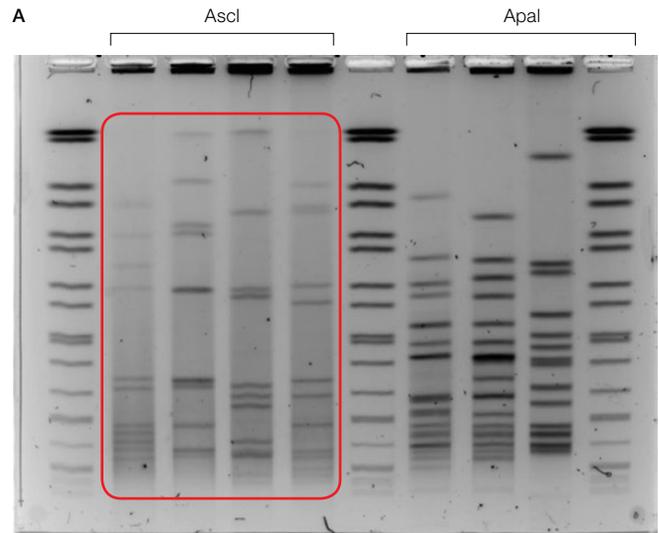
Note: Star activity can be confused with poor lysis because the results look similar. Therefore, both steps must be considered when troubleshooting the appearance of smearing with few bands on gels.

Potential Problems

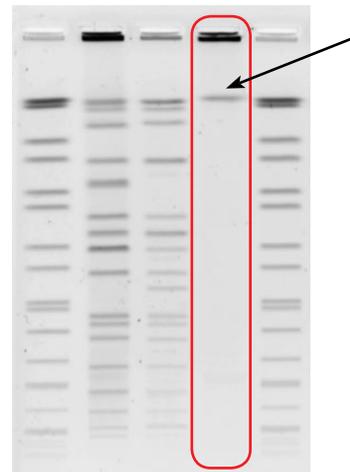
3. Some strains cannot be cut by certain enzymes because the enzyme recognition site is not present or is inaccessible. This results in the appearance of a large band at the top of the gel with no other bands or DNA smearing in the lane.

Recommendation

Digest a new plug slice to confirm that there was not an issue with that individual reaction (that is, the enzyme was omitted or the slice was not treated with the restriction mixture). If the same result is obtained, it is considered a valid pattern for that isolate.



Slices from same plugs retested. During overnight incubation of the plug slices, the enzyme AsclI exhibited star activity (A). When slices from the same plugs were incubated for 2 hr, no star activity was noted (B).



Escherichia coli 026 was digested with BlnI. A single band was confirmed to be a valid pattern for *E. coli* 026.

Using Thiourea to Type Strains

Purpose

Thiourea is used to type strains that do not yield a pattern, only a smear (that is, untypeable strains). Thiourea acts as a reducing agent and neutralizes the oxidizing species that are generated when running an electrical current through Tris-based buffers. The presence of thiourea during the run prevents degradation of modified DNA, allowing untypeable isolates to be typed.

Recommendations

Include a digested plug slice of a previously tested, thiourea-dependent isolate on the gel as a positive control. Using a positive control verifies that this is a strain problem and not a plug, reagent, or other problem.

- If the plugs are deteriorated, DNA will be left in the well and the addition of thiourea will not help
- Add 50 μ M thiourea (873 μ l of 10 mg/ml stock) directly to the electrophoresis buffer (0.5x TBE buffer) just before starting the run
- Use thiourea only when needed (with known strains or suspected serotypes) and not routinely

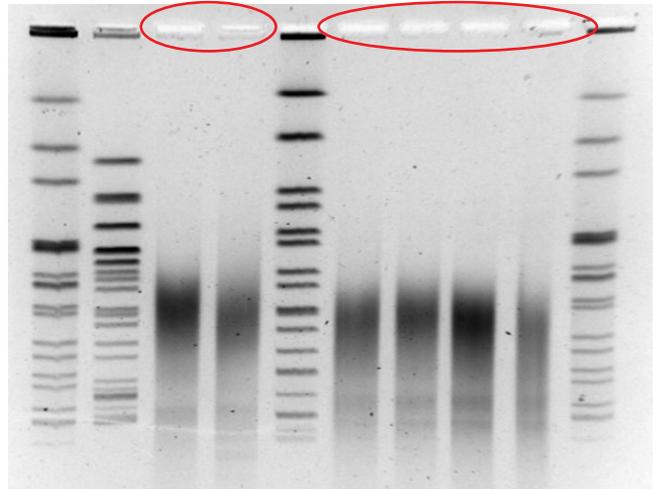
Caution: Thiourea is a mutagen and proper disposal is essential. Check with institutional hazardous waste disposal guidelines.

References

Barany F (1988). The TaqI "star" reaction: Strand preferences reveal hydrogen-bond donor and acceptor sites in canonical sequence recognition. *Gene* 65, 149–165.

University of the Basque Country. In Silico Simulation of Molecular Biology Experiments. <http://insilico.ehu.es/>, accessed November 17, 2014.

A



B



Slices from the same plugs were treated without (A) and with (B) urea and run under the same gel conditions. Lanes 3, 4, 6, 7, 8, and 9 are thiourea dependent.



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