# Allelic Typing of the Intimin Gene (*eae*) of Pathogenic *E. coli* by Fluorescent RFLP

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Intimin is a highly polymorphic protein encoded by the *eae* locus, and plays a crucial role in the attaching and effacing phenotype of diarrheagenic *Escherichia coli* (3). A number of *eae* typing schemes have either focused on allele-specific PCR amplification or conventional restriction fragment length polymorphism (RFLP) analysis (1, 4, 5), both of which have shortcomings. Allele-specific PCR amplification can lead to erroneous results if one of the primers is located in a region that is shared between two or more different alleles. Conventional RFLP analysis is limited because the sizes of restriction fragments of the highly variable 3' half of the gene may be difficult to resolve under standard electrophoretic conditions.

To address these issues, we have developed a method to quickly and accurately type the *eae* locus through the use of fluorescent RFLP (2). In this method, the entire highly variable 3' end of *eae* is amplified in a standard PCR reaction using primers that are located in the conserved central domain of *eae* and in the conserved downstream gene *escD*. The PCR amplicon (~2 kb) is then digested with one or more restriction enzymes that leave a 5' overhang, which acts as a template for the incorporation of a fluorescent dye-terminator nucleotide from a standard cycle sequencing kit. The labeled fragments are then separated on a capillary-based sequencer.

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## REFERENCES

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**Ideal timetable:**

1. Day 1 morning – set up PCR
2. Day 1 late morning / early afternoon – run PCR gel
3. Day 1 early / mid afternoon – set up restriction digest and incubate overnight
4. Day 2 morning – set up fRFLP
5. Day 2 early / mid afternoon – run fRFLP samples on CEQ

**PCR:**

1. Set up PCR so that each 25-μL reaction contains:

| | | |
|---|---|---|
| 2.50 μL | 10X buffer | |
| 2.50 μL | dNTP (2 mM each) | |
| 2.00 μL | $MgCl_2$ (25 mM) | |
| 0.50 μL | eae-F1 (10 μM) | (5'-ACTCCGATTCCTCTGGTGAC-3') |
| 0.50 μL | escD-R1 (10 μM) | (5'-GTATCAACATCTCCCGCCCA-3') |
| 0.30 μL | Taq (1.5 U) | |
| 15.70 μL | $ddH_2O$ | |
| 1.00 μL | template DNA (~50 ng/μL) | |

2. Use the following thermocycling parameters:

| Thermocycle | Temp. | Time | |
|---|---|---|---|
| Soak | 94°C | 10 min. | |
| Denature | 92°C | 1 min. | } |
| Anneal | 55°C | 1 min. | } 35 cycles |
| Extend | 72°C | 2 min. | } |
| Soak | 72°C | 5 min. | |
| Hold | 4°C | | |

**PCR gel:**

1. Make a 0.8% agarose gel (0.56 g agarose in 70 mL 1X TAE).
2. Add 3.5 μL of ethidium bromide to the molten agarose.
3. Load 3 μL of loading buffer with 5 μL of PCR product.
4. Load 1 μg of 1 kb+ ladder.
5. Run gel at ~90-110 V until the dye front has migrated about half way down the gel.

**PCR clean up and digestion:**

1. For the samples that were positive for the *eae*/*escD* PCR, treat with ExoSAP-IT to remove unincorporated dNTPs and PCR primers (5 μL of PCR product + 2 μL of ExoSAP-IT). If performing both sets of restriction digests (see below), use 10 μL of PCR product and 4 μL of ExoSAP-IT.
2. Mix thoroughly and incubate at 37°C for 15 minutes followed by 80°C for 15 minutes to inactivate the enzymes. It is best to do this step in a thermocycler. Use a 4ºC hold after the 80ºC incubation period to preserve samples.
3. Set up *Mse*I or *Ase*I / *Dde*I / *Sal*I digest cocktail so that each reaction contains:

| | | | |
|---|---|---|---|
| 0.30 μL | 100X BSA | 0.30 μL | 100X BSA |
| 0.50 μL | *Mse*I (5 units) | 0.50 μL | *Ase*I (5 units) |
| 3.00 μL | 10X NEB2 buffer | 0.50 μL | *Dde*I (5 units) |
| 19.20 μL | $ddH_2O$ | 0.25 μL | *Sal*I (5 units) |
| | | 3.00 μL | 10X NEB3 buffer |
| | | 18.45 μL | $ddH_2O$ |

4. Add 23 μL of the restriction digest cocktail to 7 μL of ExoSAP-treated PCR product.
5. Vortex briefly and spin down.
6. Incubate reactions at 37°C overnight in either a water bath or thermocycler (37°C for 1 min. followed by a 37°C hold instead of the usual 4°C hold).

**fRFLP set up and purification:**

1. Hydrate Sephadex columns **<u>two hours</u>** before setting up fRFLP reactions. (see Cycle Sequencing Protocol for specifics.) Alternatively, the Sephadex columns can be hydrated after setting up the restriction digest. Simply wrap the plates in plastic wrap and place in the refrigerator.
2. Set up a fRFLP cocktail using the white CEQ kit (not the green quick start kit) so that each reaction contains:

| | |
|---|---|
| 1.5 μL | 10X CEQ buffer |
| 0.1 μL | ddUTP |
| 0.1 μL | Taq |
| 11.3 μL | $ddH_2O$ |

3. Add 2 μL of the restriction digest to 13 μL of fRFLP cocktail.
4. Incubate samples in a thermocycler at 60°C for 1 hour. Remove Sephadex plate from refrigerator so it can warm up to room temperature (if applicable). Upon completion of this step, the Sephadex columns will be fully hydrated.
5. Purify samples with the Sephadex columns and then dry the samples (see Cycle Sequencing Protocol for specifics.)

**fRFLP rehydration and loading:**

1. Rehydrate samples in 10 μL of deionized formamide.
2. Mix 5 μL of the 600-nucleotide size standard with 360 μL of deionized formamide (this will be enough for 8 samples, adjust quantities as needed). Add 40 μL of size standard & formamide mixture to each well of the CEQ sample plate.
3. Add 2 μL of the rehydrated fRFLP samples to the CEQ sample plate. Mix samples thoroughly. This is best done by setting a multichannel pipettor to 20 μL and then pipetting up and down carefully.
4. Make sure there aren't any bubbles at the bottom of your sample wells and then add 1 drop of mineral oil to each sample of prevent dehydration.
5. Load samples on to the CEQ. (see Cycle Sequencing Protocol for specifics.)
6. Set the run and analysis methods to "fRFLP" (use "fRFLP 90" run method for *Ase*I / *Dde*I / *Sal*I digests).

fRFLP run method:

| | | |
|---|---|---|
| Capillary: | temp.: 50°C | wait for temp.: Yes |
| Denature: | temp.: 90°C | duration: 120 sec. |
| Inject: | voltage: 2.0 kV | duration: 30 sec. |
| Separate: | voltage: 4.8 kV | duration: 65.0 min. (90 min. for triple digest) |
| Pause: | 0 min. | |

fRFLP analysis method:

| | |
|---|---|
| Standard: | SizeStandard-600 |
| Std Mobility Reference: | none |
| Advanced: | Slope threshold: 100 |
| | Dye mobility calibration: NoCorrection |
| Analysis method: | Include peaks: 30% |
| | Define model: Model = Quartic, Y variable = Mobility |

7. If the sample has small peaks that are not included in the analysis, adjust the fRFLP analysis method. Change the slope threshold to 50 and the include peaks to 15%. Be sure to change these values back to the original values when the reanalysis is complete. If the modified values are used, noise peaks may be included in the analysis, which could make typing the allele more difficult.