Molecular typing by PFGE: gel production and staining, image acquisition and self-evaluation

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EURL- VTEC

Second Joint Training Course on the Use of BioNumerics Software to analyse PFGE data of STEC, *Salmonella* and *Listeria monocytogenes*

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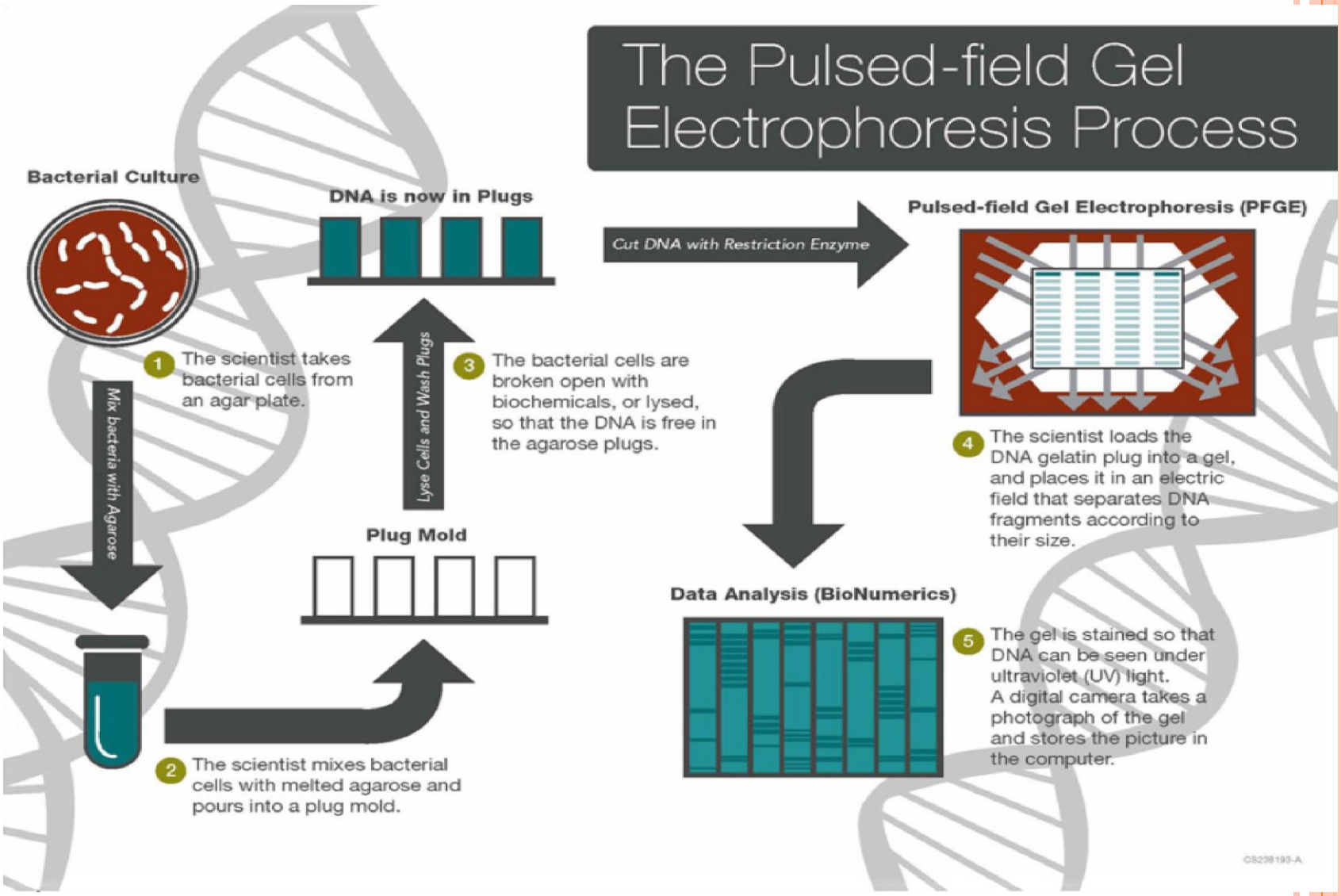
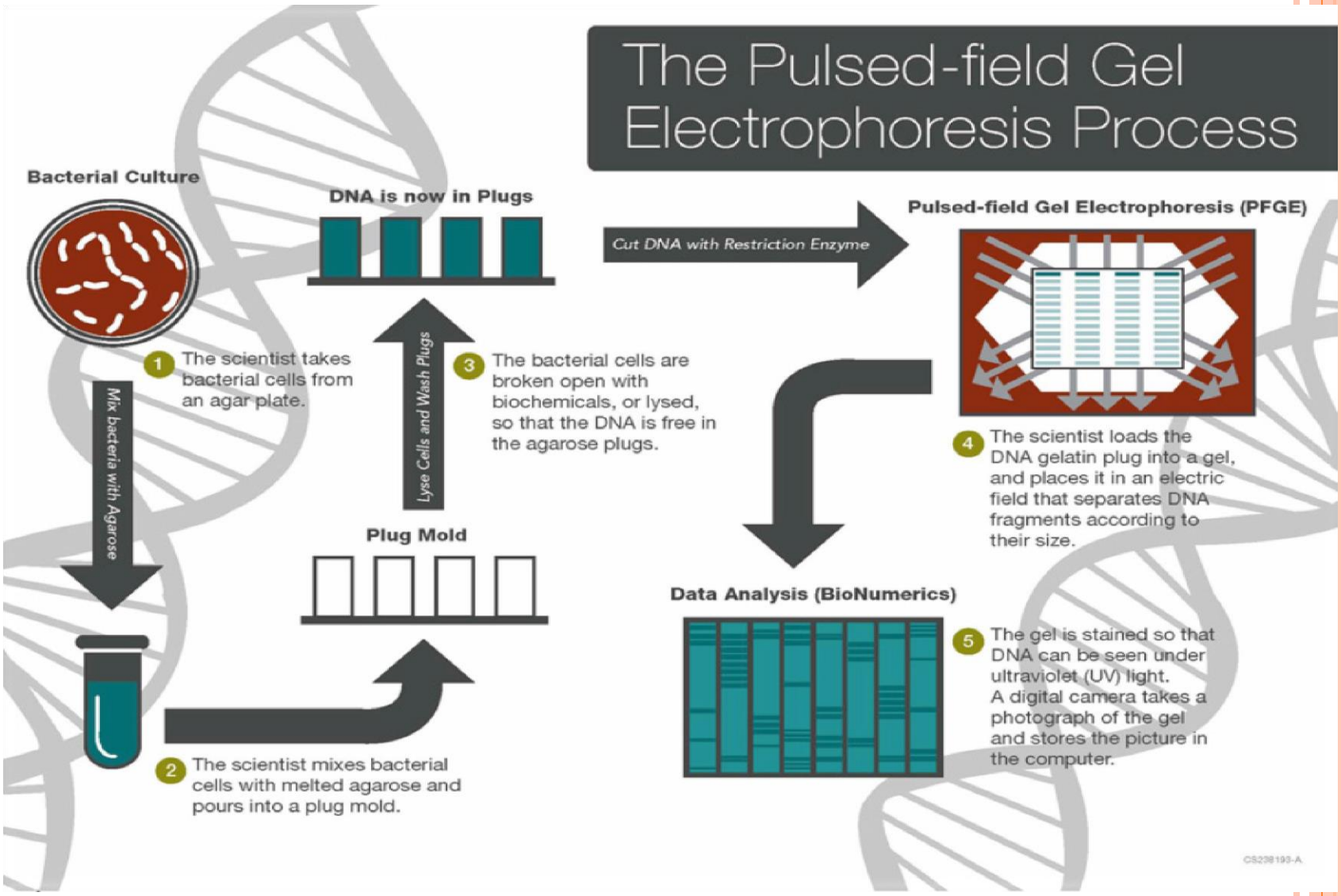
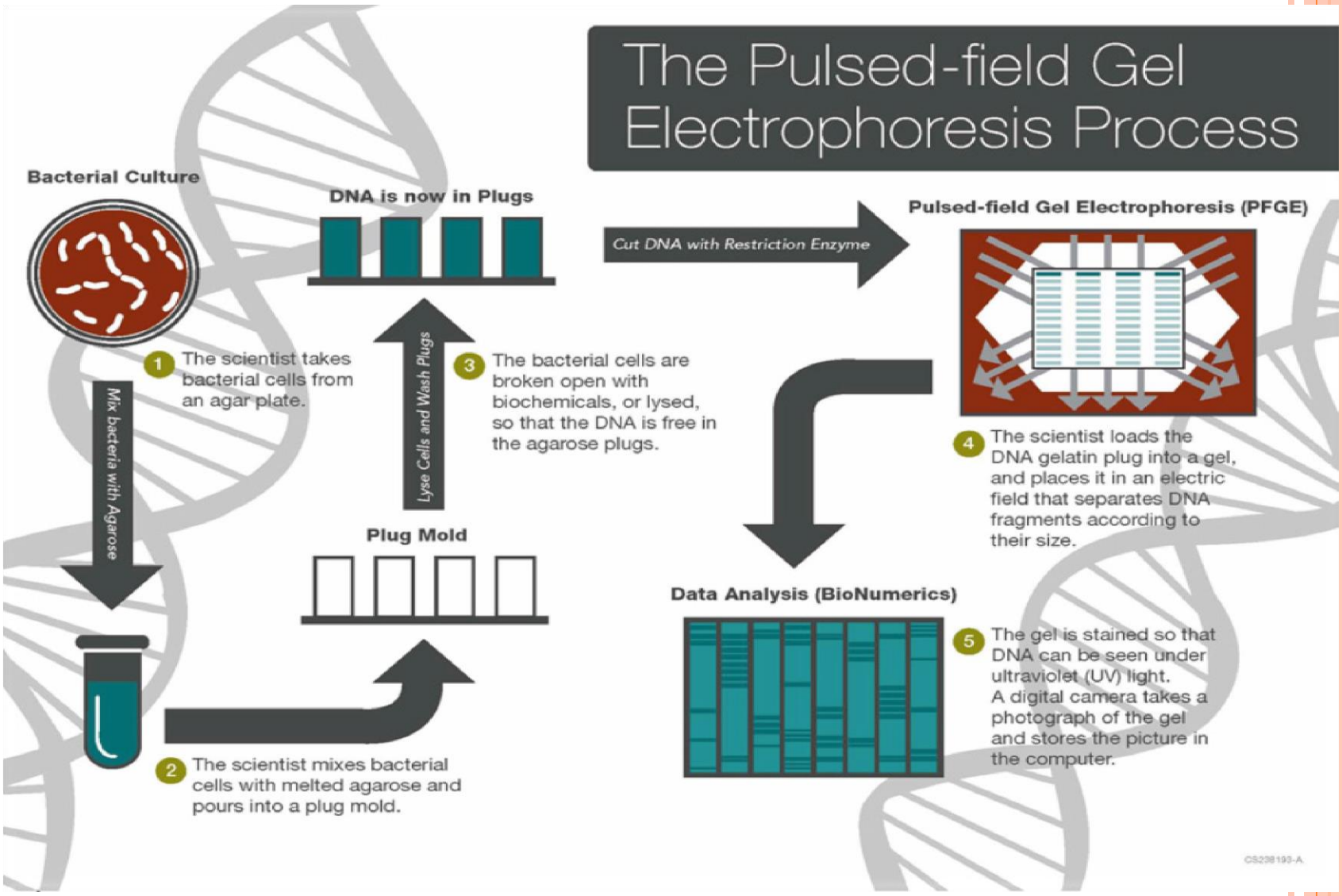
ISS – Istituto Superiore di Sanità, Roma - ITALY



- **Set up 30 years ago**

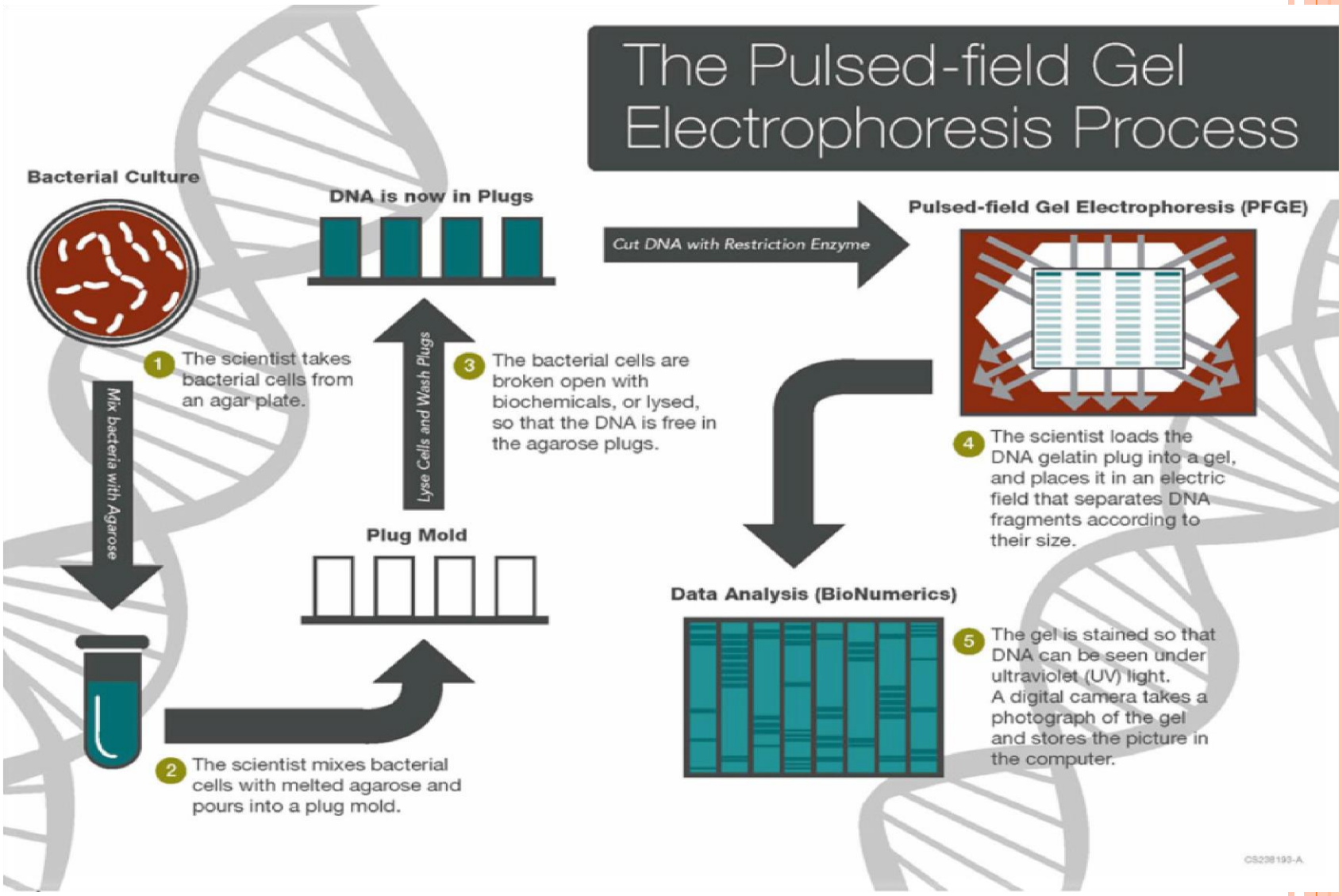


- **Separate long strands of DNA through an agarose gel by applying a pulsed electric field**

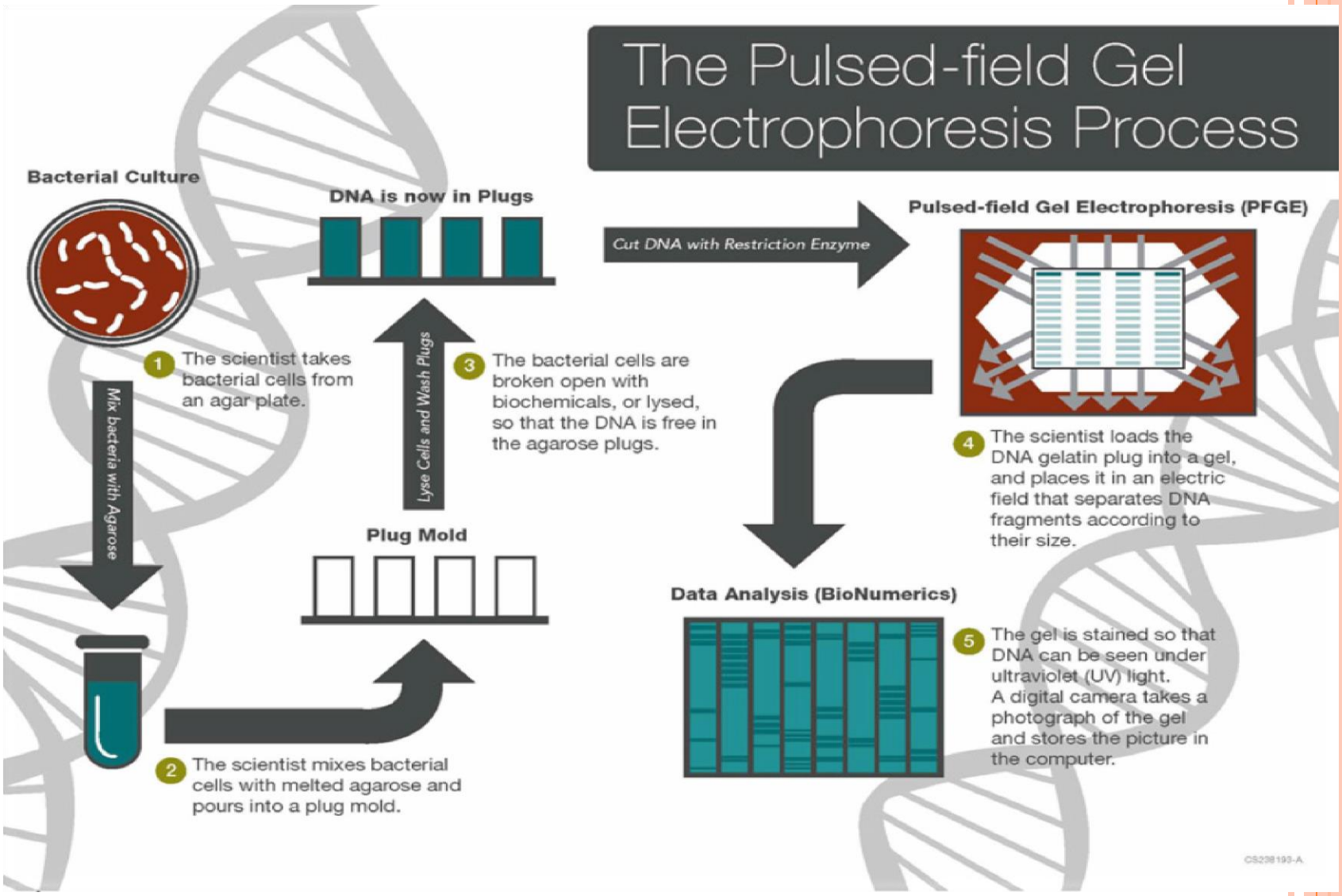


- **PFGE resolves extremely large DNA, raising from 30-50 kb to well over 10 Mb**

THE WHOLE GENOME CONTENT OF BACTERIA CAN BE SOLVED



- **The gold standard typing method commonly used for outbreak identification, surveillance and investigation for a number of important pathogens, in particular Salmonella, STEC and Listeria**



- **PulseNet International is a network dedicated to track foodborne infections world-wide- many efforts for methods standardization, including PFGE**

HEALTH AND CONSUMERS
Food

2012

http://ec.europa.eu/food/food/biosafety/salmonella/docs/vision-paper_en.pdf.

Vision paper on the development of data bases for molecular testing of foodborne pathogens in view of outbreak preparedness.

1. Background

1.1. Molecular testing

Molecular typing or microbial DNA fingerprinting has developed rapidly in recent years. Many typing methods, like PCR techniques and sequencing, have become part of routine strain characterization in many laboratories. Molecular typing provides essential tools for different surveillance purposes, such as monitoring spread of clones and strains, early detection of dispersed (international) outbreaks, and prediction of epidemic potential.

- ✓ **PFGE SOPs produced by EURLs for STEC, *Listeria monocytogenes* and Salmonella are based on the PulseNet protocol**
- ✓ **http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/704e.pdf; /702e.pdf; /703e.pdf**

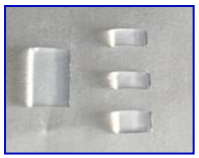
PFGE analysis at a glance…

Bacterial cell suspension

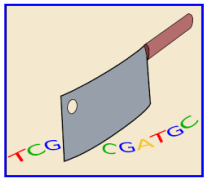
Mix with agarose

Plugs preparation

Lysis and washing



Bacterial DNA in plugs

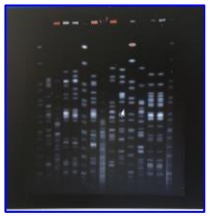


DNA restriction

plugs are loaded onto agarose gel

PFGE run

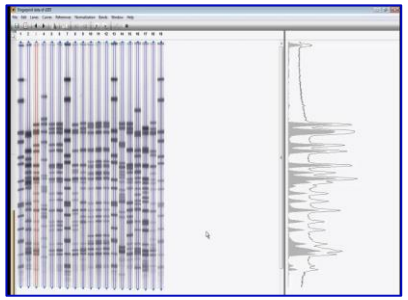
Adequate gel staning/de-staining



Gel image acquisition → Image Analysis

BACTERIAL DNA IS EMBEDDED IN AGAROSE PLUGS TO AVOID SHEARING AND RESTRICTED WITH RARE-CUTTING ENDONUCLEASE

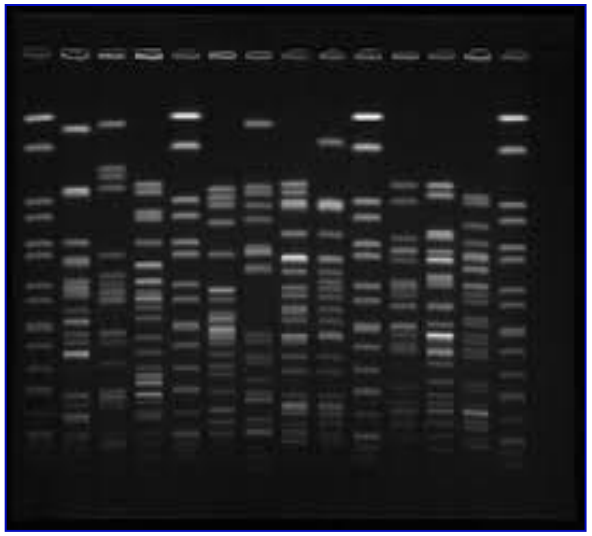
PARAMETERS AFFECTING THE GEL RUN: **BUFFER**, **AGAROSE**, **ANGLE** OF THE FIELD, **VOLTAGE**, **TIME SWITCH, TEMPERATURE**, **TIME**



A TIFF image of the PFGE gel is required for the analyses with the BioNumerics software

Tag Image File Format is a rich format for pixel based raster image data from many sources

The production of a good PFGE TIFF depends on all the steps mentioned



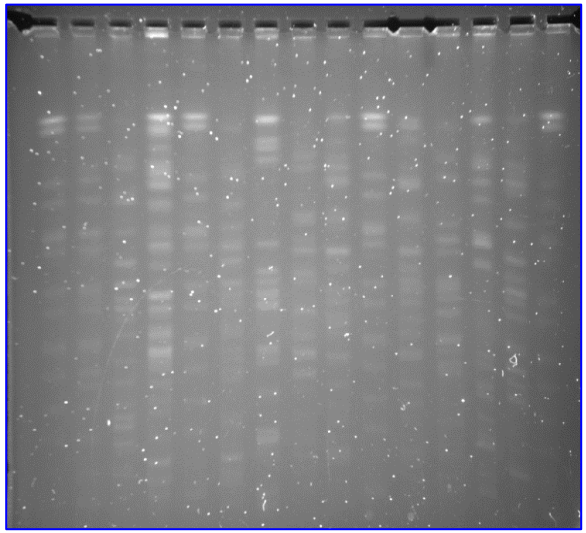
DNA preparation

Proper DNA restriction

PFGE run

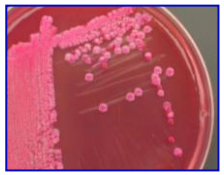
Proper Gel Staining/De-staining

Good Gel Image documentation



Quality Assessment of PFGE images

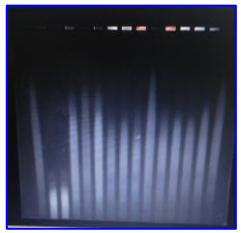
Quality of DNA

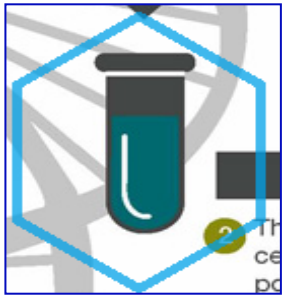


Start with pure, fresh bacterial cultures

If cultures are not pure, it is not possible to obtain a single pattern

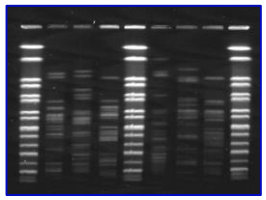
If cultures are not fresh, bacteria may produce endonucleases resulting in DNA degradation

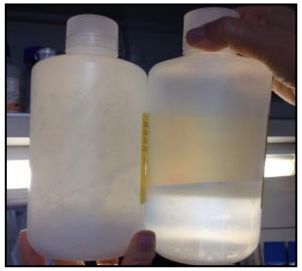




Standardize the bacterial suspensions

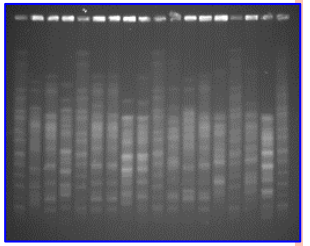
If different concentrations of bacterial cells are used each lane will have darker and lighter bands- difficult analysis





Use sterile, freshly prepared solutions

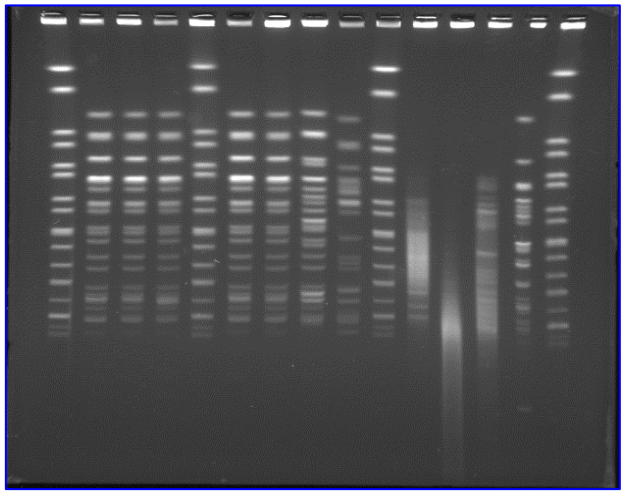
The concentration may vary in old solutions, nucleases may be present affecting the DNA integrity



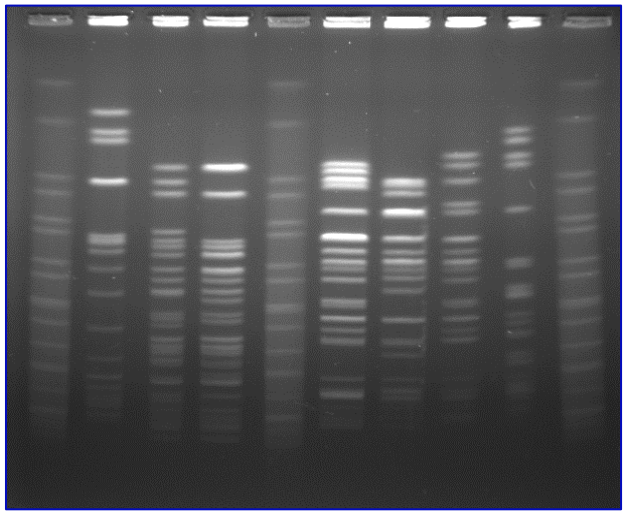
Wash thoroughly the plugs after lysis

Proteinase K must be completely removed - it could inhibit restriction

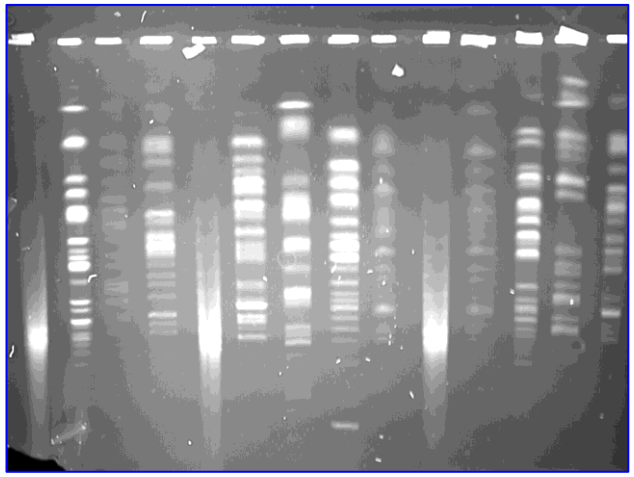
Some examples….



Degradation of DNA



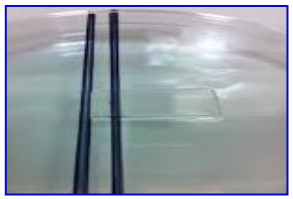
Different intensity of DNA in each lane



Many critical points could have been at the origin of the problem, including:

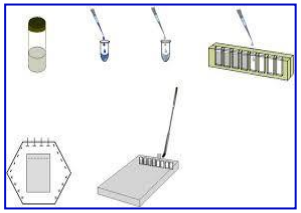
- The use of not freshly grown cultures.
- Plugs prepared using solutions that were not nuclease free.
- Presence of nucleases in the restriction buffers

DNA restriction



Use appropriate sized-plugs

When using 15 or more -well DNA comb cut the plugs



Pre-restrict the plugs

Letting the plugs equilibrate with the restriction buffer will improve digestion

Use BSA

BSA is a stabilizer of restriction endonucleases, so its use enhances the enzymes activity

MAKE SURE THAT THE PLUGS ARE COVERED WITH PRE-RESTRICTION/RESTRICTION BUFFER

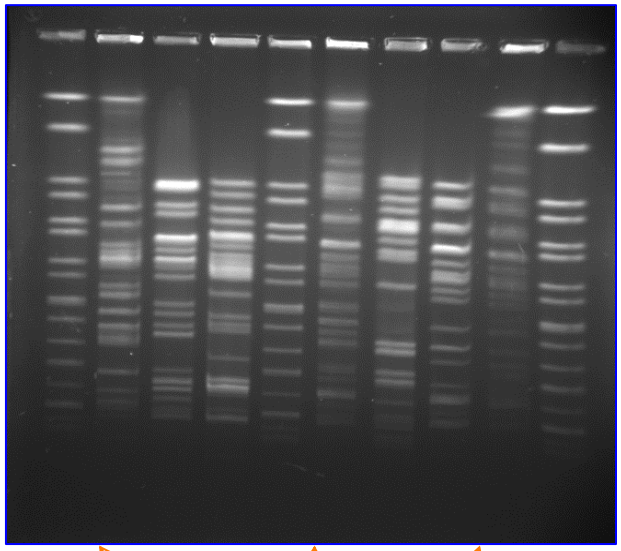
Use the appropriate amount of Enzyme

Using less units of restriction endonuclease may result in incomplete digestion

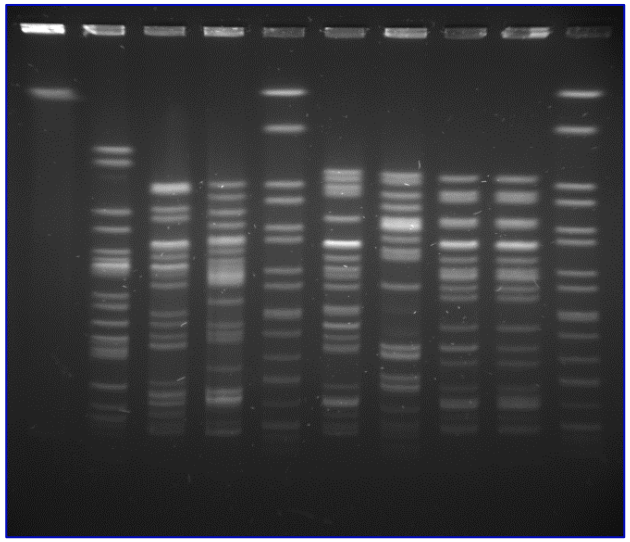
Don't let restriction go too far

Too long restriction of DNA may result in degradation

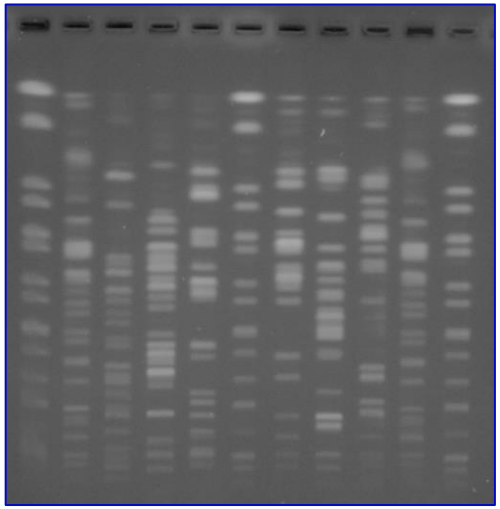
A few examples….



Partial restriction



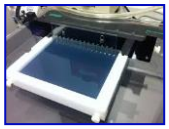
No restriction!!!!



Many partially restricted fragments

PFGE run

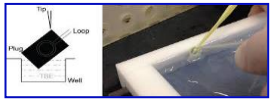
TBE Buffer must be prepared fresh and used only once



Let the gel solidify completely before removing the comb

Place the gel form on a levelling table and adjust until perfectly levelled

Cover the gel for preventing that dust can go into it



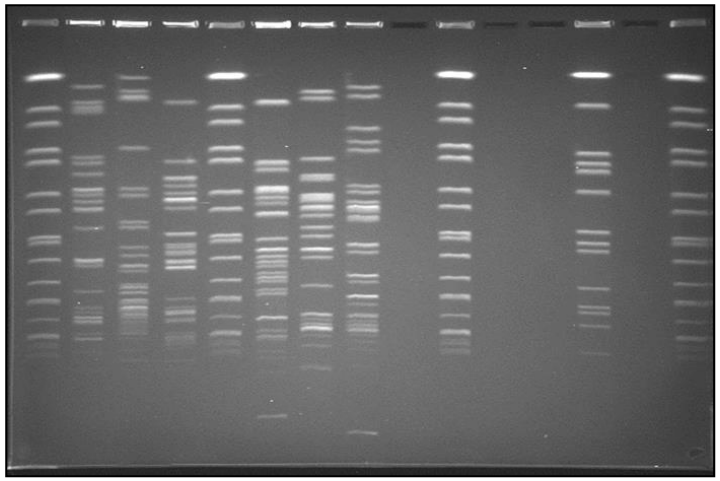
Carefully load the plugs into the wells and make sure that they don't move during the run, avoiding plugs damaging

Run the gel with the correct running conditions (switch time)

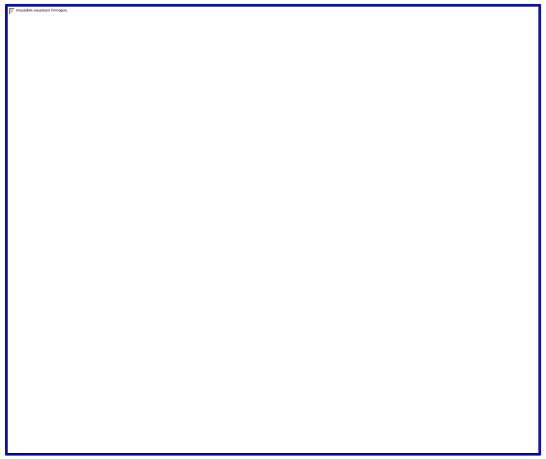
Temperature and current are crucial

Check the quality of the water used for the electrophoresis (conductivity 18,2mΩ/cm) and the absence of any contaminants

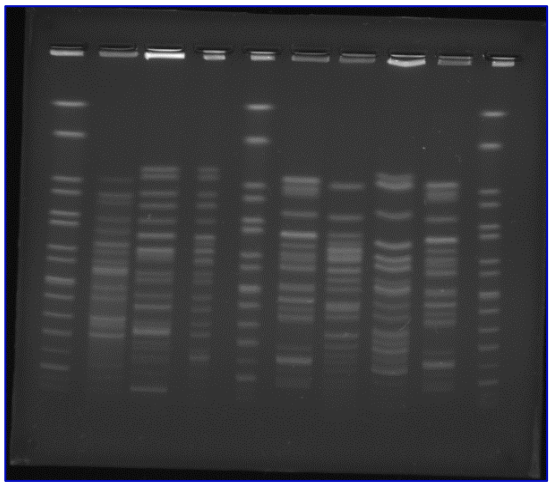
Examples



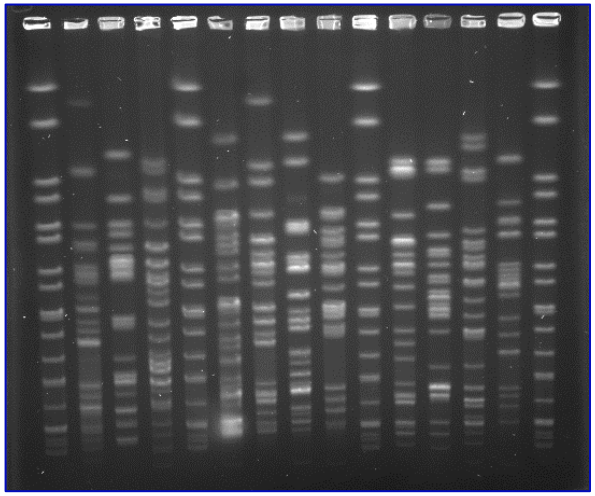
Non-O157 running conditions for STEC/XbaI profiling



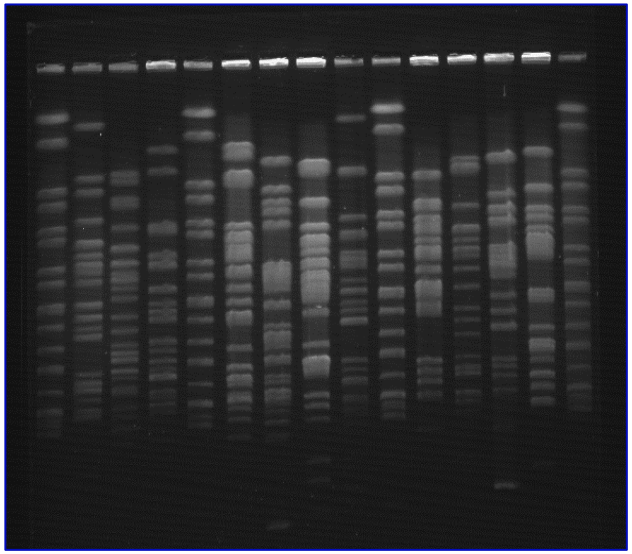
Plug damaged



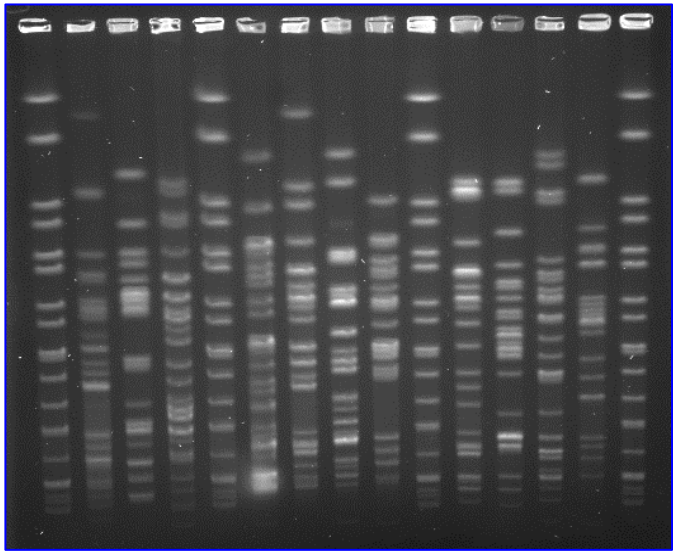
«Smile» effect



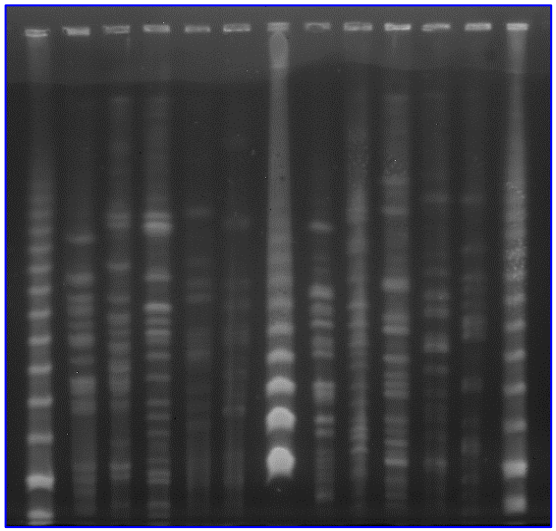
The pattern is not sharp in a few lanes



Movement of the plugs

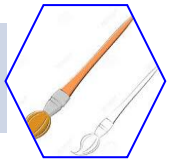


Blurred bands in some lanes



DNA degradation, too long electrophoresiss time, wrong Molecular Size marker, …

Gel Staining/De-staining



Stain the gel in freshly prepared 1 µg/ml EtBr solution

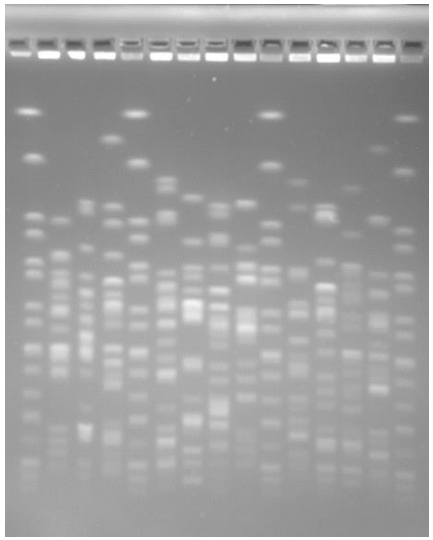
Do not stain for more than 20 minutes
this would make it difficult to remove background

De-stain the gel in pure water
More than one time by changing the water every 20-30 minutes

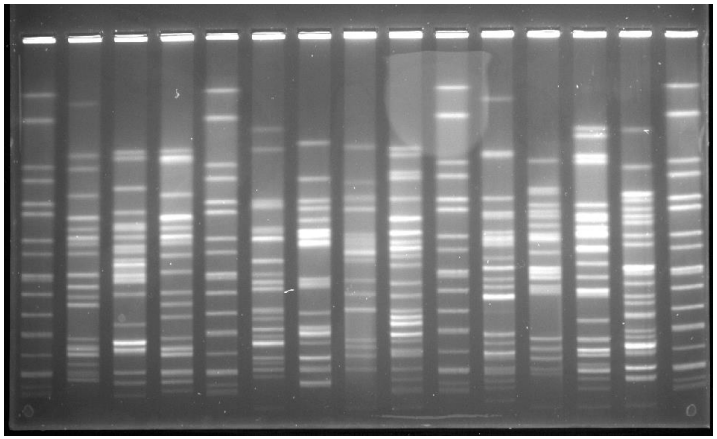
Staining and de-staining shall be done in clean boxes with slow agitation

PROPER DE-STAINING HELPS IN AVOIDING HIGH BACKGROUND NOISE

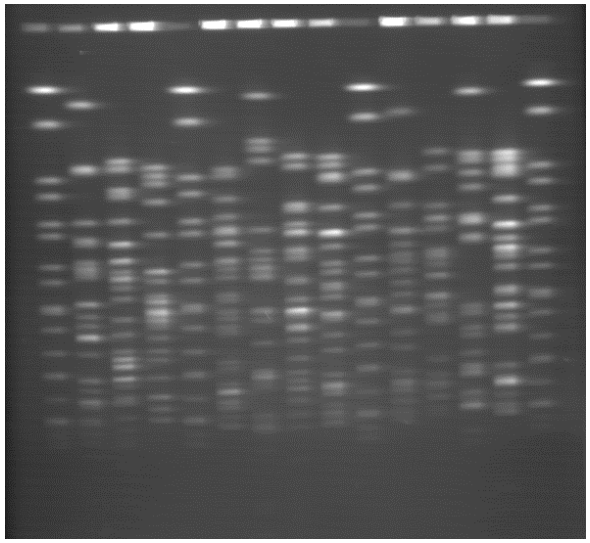
Examples



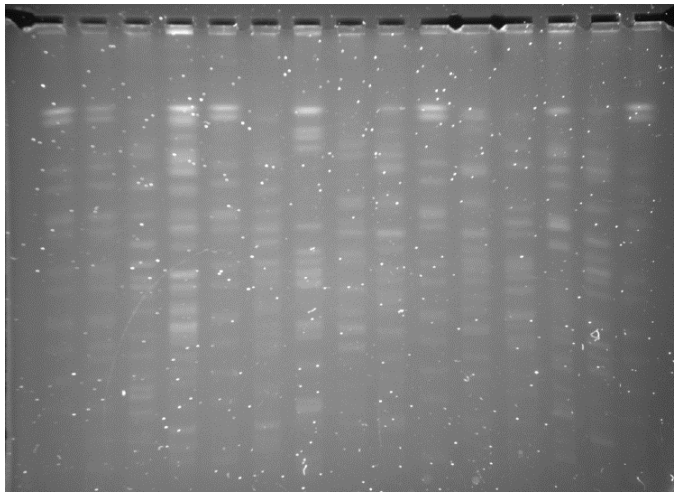
Very high background



High background, lot of stains



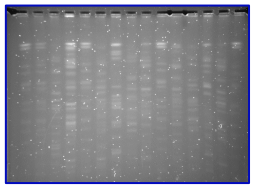
Out of focus of the bands



Exhausted staining solution.
Many debris are present all over the gel.

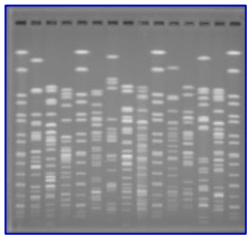
Gel Image acquisition

Capture the whole gel in the image (from top to bottom)



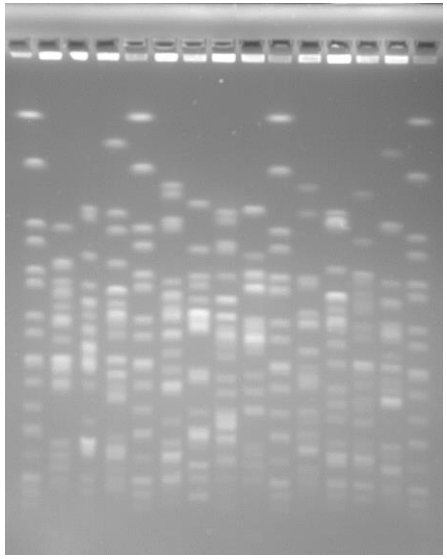
The image must be on focus

Avoid over-exposure as it would make difficult the following analysis

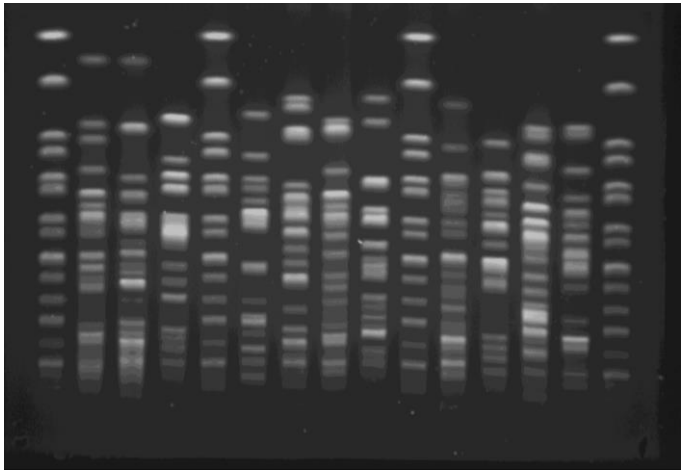


More than one image aquisition may be done, but only one must be further used for the analysis

Examples



Overexposure



Out of focus

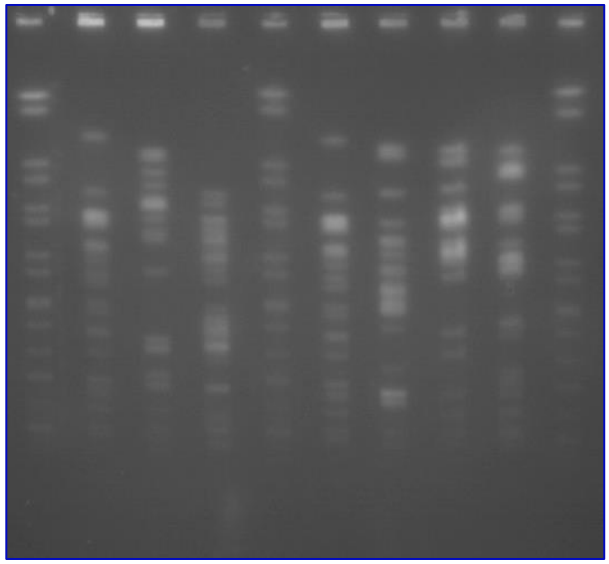
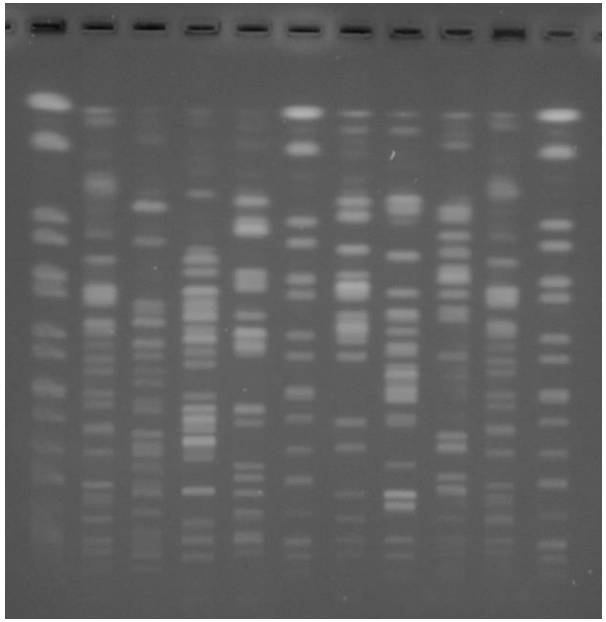


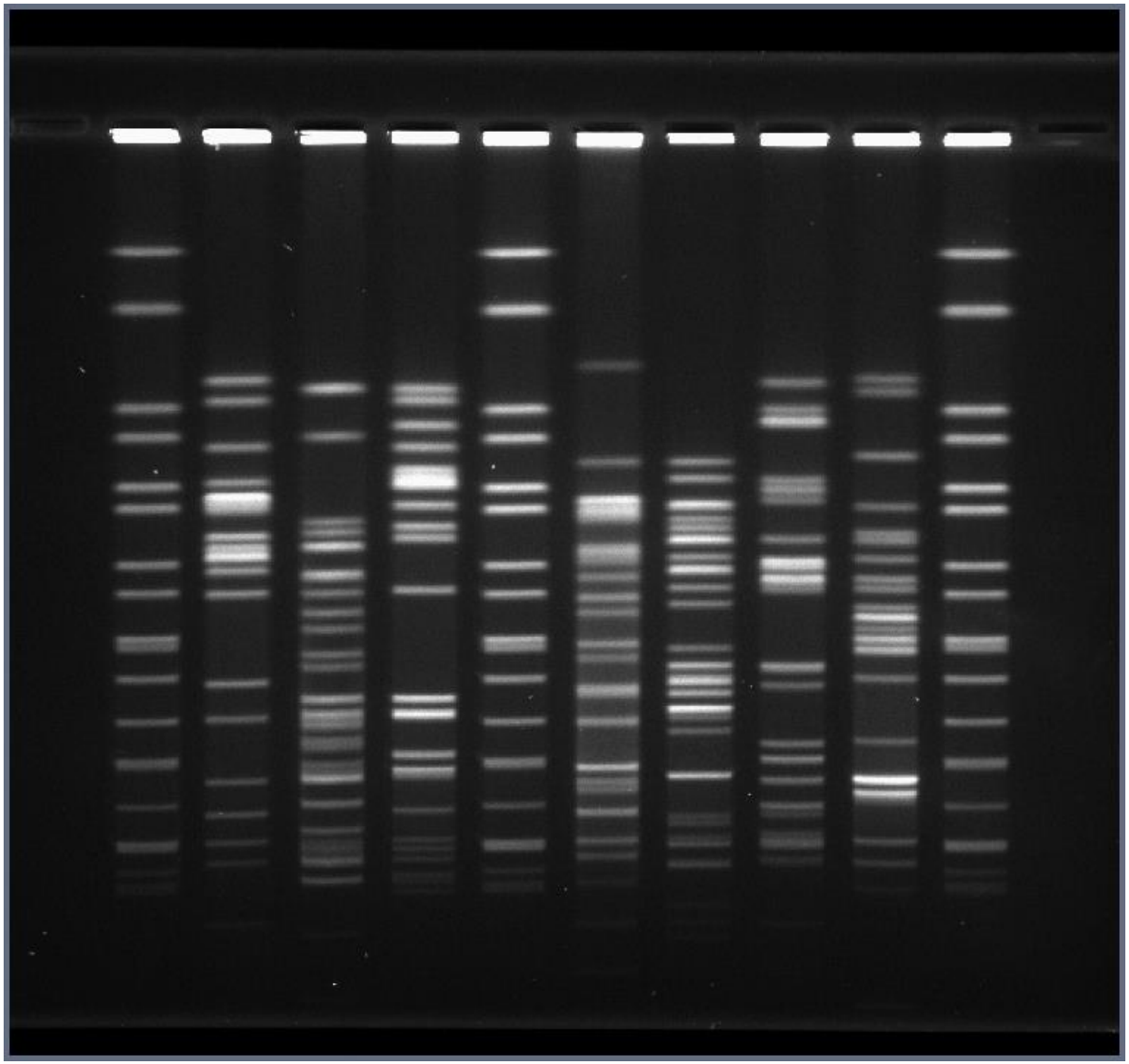
Image appears fuzzy and out of focus. The image acquisition process should be largely improved. An increase in the de-staining time might be of help in decreasing the background. The use of exhausted staining solutions should be avoided.
The majority of the PFGE profiles seem to be poorly defined, particularly in the central part of the gel. The bands are fuzzy and very difficult to visualize.
Too prolonged exposure on the UV lamp can damage the DNA



Many partially restricted fragments are visible in the upper part of all lanes.
The distribution of band intensity among lanes is not uniform. The standardization of the bacterial cells concentration in the cultures needs improvement.
The bands are fuzzy and difficult to visualize.

Self-evaluation of quality assessment of TIFF files

- ✓ The gel image should fill the entire window screen (without cutting off wells or lower bands, the end of the gel must be visible in the TIFF image).
- ✓ The gel image should be in focus with no over-exposure of the bands.
- ✓ The bottom band of the standard must be 1-1.5 cm from the bottom of gel.
- ✓ The resolution of the images must be an 8 bit uncompressed gray-scale TIFF image.
- ✓ The intensity of the bands should be approximately the same in each lane.
- ✓ The bands should be clear and distinct all the way to the bottom of the gel; some band distortion can be accepted, but it should not interfere with the analysis.
- ✓ The gel background should be mostly clear, not affecting the analysis.
- ✓ DNA degradation should be avoided, in order to product clear bands.



The BioNumerics Software: database creation, experiment type, import of TIFF files, and setting up experiments

A SIX STEPS PROCESS:

- **STEP 1-** Create a new database
- **STEP 2-** Create the experiment type (Fingerprint)
- **STEP 3-** Import the TIFF files into the Database
- **STEP 4-** Analyze a TIFF:
  1. Convert a TIFF to Gel Strips
  2. Define Curves
  3. Normalize the gel
  4. Find Gel Bands
- **STEP 5-** Link Lanes to Database Entries
- **STEP 6-** Add information on the Isolates (virulence genes, serogroups etc…)

Coffee break now!