LABORATORY MANUAL

ON

METHODS IN MOLECULAR BIOLOGY

Contents

In-vitro Amplification of DNA by Polymerase Chain Reaction (PCR)

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Polymerase chain reaction (PCR) is a technique which is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. This technique can be used to identify disease-causing viruses and/or bacteria, a deceased person, or a criminal suspect.

In order to use PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA (may be a gene or any sequence). One need not know the DNA sequence in-between. The nucleotide sequences of many of the genes and flanking regions of genes of many different organisms are known. We also know that the DNA of different organisms is different. While some genes may be the same, or very similar among organisms, there will always be genes whose DNA sequences differ among different organisms - otherwise, would be the same organism (e.g., same virus, same bacterium, an identical twin; therefore, by identifying the genes which are different, and therefore unique, one can use this information to identify an organism.

Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases.

Components of PCR:

Oligonucleotides (primer pairs)

Oligonucleotides used for priming the PCR should be at least 16 nucleotides and preferably 20-24 nucleotides in length. Primer pairs can be commercially purchased in the form of desalted or HPLC purified. Reconstitute the primers with sterile distilled water to the required concentration (usually 10mM).

Usually, primers are used at a final concentration of 1-4 mM in PCR assay. This is usually sufficient for 30-35 cycles of amplification. The presence of higher concentrations of primers can cause priming at ectopic sites, with consequent amplification of undesirable non-target sequences. Conversely, the PCR is extremely inefficient when the concentration of primers is limiting. If the yield of amplified product is poor or if contamination by non-target sequences is unacceptably high, set up a series of control experiments to determine the

minimum amount of the two primers required to generate the desired size of amplified product keeping the template DNA concentration constant. After standardization, many pairs of primers can be used in a single reaction as a multiplex PCR to detect different target genes simultaneously.

PCR Buffer

The standard PCR buffer contains 50mM KCl, 10mM Tris-HCl (pH 8.3 at room temperature) and 1.5 mM $Mgcl_2$.When the reaction performed at 72°C (annealing temperature), the pH of the reaction drops by more than a full unit (pH 7.2). The presence of divalent cations is also critical. Magnesium ions are superior to manganese, and calcium ions are ineffective. In a standard buffer, the optimal concentration of Mg++ is quite low (1.5mM), it is important that the preparation of template DNA does not contain high concentrations of chelating agents such as EDTA or negatively charged ionic groups such as phosphates. The template DNAs/ bacterial cultures should therefore be dissolved/ suspended in sterile water.

The concentration of Mg++ should be optimized whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is altered. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affect the concentration of Mg++. PCR buffer are generally supplied in 10x concentrations with or without $MgCl_2$ along with Taq polymerase.

Taq *DNA polymerase*

Two forms of Taq polymerase are available; the native enzyme purified from the thermophilic bacterium *Thermus aquaticus* and a genetically engineered form of the enzyme synthesized in E. coli (rTaq/Ampli TaqTM).

Both forms of the polymerase carry 5/ 3/ polymerization-dependent exonuclease activity, but lack a 3/ → 5/ exonuclease activity. The properties of the two polymerases are essentially identical.

This enzyme activity can persist extended incubation even at 95°C and hence not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle. In addition, because of annealing and extension of primers can be carried out at elevated temperatures, mispriming is greatly reduced. This result in substantial improvements in the specificity and yield of the amplification reaction and in the size of the amplification product. Approximately 1 unit of the enzyme is required to catalyze a typical reaction. Addition of excess enzyme may lead to amplification of non-target sequences. To ensure high specificity of the synthesized products the addition of Taq DNA polymerase is withheld until the reaction temperature is at 80°C. This method has been named as "Hot start".

Deoxyribonucleoside triphosphates (dNTP)

It is a mixture of dATP, dGTP, dCTP and dTTP at saturating concentration of 200 mM for each. It is also commercially available. pH of dNTP should be 7.0. Sometimes commercially available dNTPs are not adjusted to pH 7.0.

Target sequences (Template DNA)

DNA containing the target sequences can be added to the PCR mixture in a single or double stranded form. The size of the DNA is not a critical factor; however, target sequences are amplified slightly less efficiently when they are carried in closed circular DNAs rather than in linear DNAs. The concentration of target sequences in the template DNA varies from 0.001ng to 1.0ng/µl.

The PCR is commonly carried out in a reaction volume of 10-200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

Basic composition of 10X PCR Buffer (pH 7.0)

500 mM KCl
100 mM Tris Cl (pH 8.3 at room temperature)
15 mM $MgCl_2$
0.1% gelatin

Reagents required:

1. Sterile distilled water
2. 100 mM $MgCl_2$
3. 10X PCR buffer
4. 2mM dNTP mix
5. 10µM oligonucleotide primers; forward and reverse (10 pmol/ml in sterile H_2O; store at – 20 °C)
6. Template DNA
7. 5 U/ml Taq DNA polymerase
8. Mineral oil (if the PCR instrument is not provided with heated hood)
9. 2% agarose

Equipments

1. Pipette
2. Plastic wares including microfuge tubes (1.5 and 2.0 ml), and tips
3. Microcentrifuge apparatus

4. Gel documentation system or UV transilluminator
5. Aspirator
6. Shaker water bath
7. Automated thermal cycler
8. Electrophoresis apparatus

Procedure:

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent cations and dNTPs in the reaction and the melting temperature (Tm) of the primers.

Initialization step: This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98 °C for 20-30 seconds. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step: The reaction temperature is lowered to 50-65 °C f or 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used. Taq polymerase has its optimum activity at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the

amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation: This single step is occasionally performed at a temperature of 70-74 °C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4-15°C for an indefinite time may be employed for short term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

Important notes before starting:

- PCR is capable of amplifying as little as single molecule of DNA, precautions should be taken to guard against contamination of the reaction mixture with trace amounts of DNAs that could serve as templates. PCR is generally performed in a dedicated sterile place (preferably in a laminar flow hood) using positive displacement pipettes equipped with disposable tips. Use autoclaved high quality distilled water, sterile tips and microfuge tubes.

- Before opening microfuge tubes containing reagents used in PCR, centrifuge them briefly. This will reduces the possibility of contamination of gloves or pipetting devices. While using the Taq polymerase, it is recommended to use the 10X PCR buffer provided along with the enzyme.

- Add all components of the reaction to the microfuge tube before adding the template DNA. The choice of the PCR tubes (0.2 or 0.5 ml) depends on the type of block in the PCR instrument. If the $MgCl_2$ is not added in the 10X PCR buffer, use the one provided by the manufacturer to the recommended concentration

- When adding the template DNA to a PCR mixture, take care not to create aerosols that could contaminate other reactions. Keep all the tubes closed that are not in immediate use. Change gloves after handling tubes containing template DNA.

- Include control i.e., a PCR tube that contains all the components of the PCR except the template DNA.

- Mouth pipetting is not recommended for handling reagents used in the PCR.

Applications of PCR:

Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or Northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism. Bacterial colonies (E. coli) can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing. This technique may also be used to determine evolutionary relationships among organisms.

Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar.

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Real-time PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

PCR in diagnosis of diseases

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000-fold higher than that of other methods.

PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

Viral DNA can likewise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in treatment. The amount of virus ("viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques.

Variations on the basic PCR technique

- ***Allele-specific PCR:*** A diagnostic or cloning technique based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

- ***Assembly PCR or Polymerase Cycling Assembly (PCA):*** Artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.

- ***Asymmetric PCR:*** Preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher

melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

- ***Helicase-dependent amplification:*** Similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

- ***Hot start PCR:*** A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95°C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.
- ***Intersequence-specific PCR (ISSR):*** A PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

- ***Inverse PCR:*** It is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.

- ***Ligation-mediated PCR:*** Uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA footprinting.

- ***Methylation-specific PCR (MSP):*** Developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

- ***Miniprimer PCR:*** Uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.

- ***Multiplex Ligation-dependent Probe Amplification (MLPA):*** Permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR.

- ***Multiplex-PCR***: It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

- ***Nested PCR:*** It increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

- ***Overlap-extension PCR or Splicing by overlap extension (SOE) :*** A genetic engineering technique that is used to splice together two or more DNA fragments that contain complementary sequences. It is used to join DNA pieces containing genes, regulatory sequences, or mutations; the technique enables creation of specific and long DNA constructs.
- ***Quantitative PCR (qPCR):*** Used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. qPCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative real-time PCR has a very high degree of precision. QRT-PCR (or QF-PCR) methods use fluorescent dyes, such as SYBR Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RTPCR (Real Time PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR (see below), often used in conjunction with Q-PCR.

- ***Reverse Transcription PCR (RT-PCR):*** It is used for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be

used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (Rapid Amplification of cDNA Ends).

- ***Solid Phase PCR:*** Encompasses multiple meanings, including Polony Amplification (where PCR colonies are derived in a gel matrix, for example), Bridge PCR (primers are covalently linked to a solid-support surface), conventional Solid Phase PCR (where Asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced Solid Phase PCR (where conventional Solid Phase PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal 'step' to favour solid support priming).

- ***Thermal asymmetric interlaced PCR (TAIL-PCR):*** For isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.

- ***Touchdown PCR (Step-down PCR):*** A variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (35°C) above the T m of the primers used, while at the later cycles, it is a few degrees
  (35°C) below the primer T m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.

- ***PAN-AC:*** Uses isothermal conditions for amplification, and may be used in living cells.

- ***Universal Fast Walking***: For genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (using only one gene specific primer and one general primer — which can lead to artefactual 'noise') by virtue of a mechanism involving lariat structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends), 5'RACE LaNe and 3'RACE LaNe.

*Information downloaded from www.wikipedia.org has been used to compile this manuscript.

Primer Designing for PCR

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Polymerase chain reaction (PCR) is widely accepted as one of the most important inventions of the 20th century in molecular biology. Small amounts of the genetic material can now be amplified to be able to identify and manipulate DNA, detect infectious organisms, detect genetic variations including mutations and numerous other tasks.

PCR involves three steps: denaturation, annealing and extension. First, the genetic material is denatured, converting double-stranded DNA molecules to single strands. The primers are then annealed to the complementary regions of the single-stranded molecules. In the third step, they are extended by the action of the DNA polymerase. All these steps are temperaturedependent and the common choice of temperatures is 940C, 600C and 700C respectively.

Good primer design is essential for successful PCR. The following is a brief description of the important considerations for designing a primer:

1. **Primer length:** It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature.

2. **Melting temperature:** Melting temperature (Tm) is defined as the temperature at which one half of the DNA duplex will dissociate to become single-stranded and indicates the duplex stability. Primers with melting temperatures in the range of 55-650C generally produce the best results. Primers with higher melting temperatures have a tendency for secondary annealing. The GC content of the sequence gives a fair indication of the Tm.

3. **Primer annealing temperature:** The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high Tm produces insufficient primer-template hybridization resulting in low PCR product yield. Too low Tm may possibly lead to non-specific products caused by a high number of base pair mismatches. Mismatch tolerance is found to have the strongest influence on PCR specificity.

4. **GC content:** The GC content (the number of guanine and cytosine bases in the primer as a percentage of the total bases) of primer should be 40-60%.

5. **GC Clamp:** The presence of G or C bases within the last five bases from the 3/ end of primers (GC clamp) helps to promote specific binding at the 3’ end due to the stronger bonding of G and C bases. More than 3 G’s or C’s sh ould be avoided in the last 5 bases at the 3’ end of the primer.

6. **Secondary structures:** Presence of the secondary structures produced by intermolecular or intra-molecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction.

i) Hairpins: It is formed by intra-molecular interaction within the primer and should be avoided. Presence of hairpins at the 3' end most adversely affects the reaction.

ii) Self Dimer: is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield.

iii) Cross Dimer: Cross dimers are formed by intermolecular interaction between sense and anti-sense primers, where they are homologous.

7. **Repeats:** A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can misprime. For example: ATATATAT. A maximum number of di-nucleotide repeats acceptable is 4 di-nucleotides.

8. **Runs:** Primers with long runs of a single base should generally be avoided as they can misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted is 4 bp.

9. **Avoid Template secondary structure:** A single stranded Nucleic acid sequences is highly unstable and fold into conformations (secondary structures). The stability of these template secondary structures depends largely on their free energy and melting temperatures(Tm).

10. **Avoid Cross homology:** To improve specificity of the primers it is necessary to avoid regions of homology. Primers designed for a sequence must not amplify other genes in the mixture. Commonly, primers are designed and then BLASTed to test the specificity.

Parameters for Primer Pair Design:

1. **Amplicon Length:** The amplicon length is dictated by the experimental goals. For qPCR, the target length is closer to 100 bp and for standard PCR, it is near 500 bp. If you

know the positions of each primer with respect to the template, the product is calculated as: Product length = (Position of antisense primer-Position of sense primer) + 1.

2. **Product position:** Primer can be located near the 5' end, the 3' end or anywhere within specified length. Generally, the sequence close to the 3' end is known with greater confidence and hence preferred most frequently.

3. **Tm of Product:** Melting Temperature (Tm) is the temperature at which one half of the DNA duplex will dissociate and become single stranded. The stability of the primertemplate DNA duplex can be measured by the melting temperature (Tm).

4. **Optimum Annealing temperature (Ta Opt):** The formula of Rychlik is most respected. It usually results in good PCR product yield with minimum false product production.

Ta Opt = 0.3 x(Tm of primer) + 0.7 x(Tm of product) -
25 Where,
Tm of primer is the melting temperature of the less stable primer-template pair Tm of product is the melting temperature of the PCR product.

5. Primer Pair Tm Mismatch Calculation: The two primers of a primer pair should have closely matched melting temperatures for maximizing PCR product yield. The difference of 5^{o}C or more can lead no amplification.

Summary:

1. Design your PCR primers to be 18-30 oligo nucleotides in length. The longer end of this range allows higher specificity and gives you space to add restriction enzyme sites to the primer end for cloning.

2. Make sure the melting temperatures (Tm) of the primers used are not more than 5°C different from each other. You can calculate Tm with this formula: Tm = 4(G + C) + 2(A + T) °C

3. Aim for a Tm between 55 and 65°C for each primer over the region of hybridization

4. Use an annealing temperature (Ta) of 5 to 10°C lower than the Tm.

5. The GC content of each primer should be in the range of 40-60% for optimum PCR efficiency.

6. Try to have uniform distribution of G and C nucleotides, as clusters of G's or C's can cause non-specific priming.

7. Avoid long runs of the same nucleotide.

8. Check that primers are not self-complementary or complementary to the other primer in the reaction mixture, as this will encourage formation of hairpins and primer dimers and

will compete with the template for the use of primer and reagent.

9. If you can, make the 3′ end terminate in C or A, as the 3′ is the end which extends and neither the C nor A nucleotide wobbles. This will increase the specificity.

10. You can avoid mispriming by making the 3′ end slightly AT rich.

11. Use the right software. Using the right software is a great way to automate these steps and minimize errors, especially when you have to design primers for many sequences.

References:

http://rothlab.ucdavis.edu/protocols/PrimerDesign.html
http://www.biochem.ucl.ac.uk/bsm/nmr/protocols/protocols/oligo.html
http://www.protocol-online.org/prot/Molecular_Biology/PCR/PCR_Primer/
http://www.mcb.uct.ac.za//pcroptim.html

Repetitive Sequence-Based PCR (rep-PCR)

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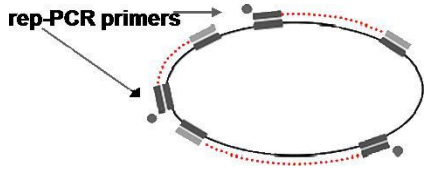
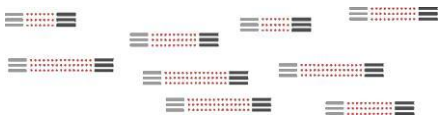
Bacterial genomes contain multiple repetitive sequences in the intergenic regions at multiple sites dispersed throughout its length. Such blocks of non-coding, repetitive sequences can serve as multiple genetic targets for oligonucleotide probes, enabling the generation of unique DNA profiles or fingerprints for individual bacterial strains. The use of defined primers for polymerase chain reaction (PCR) amplification of interspersed repetitive DNA elements present at distinct locations in prokaryotic genomes is referred to as repetitive element sequence based-polymerase chain reaction (rep-PCR). These unique "bar codes" or DNA fingerprints define each bacterial genome without the need for measuring gene expression or enzyme function. Genotypic or molecular approaches differ with respect to the level of resolution of individual bacterial species or strains into distinct categories.

Current data supports that repetitive DNA comprises a substantial portion of the microbial genomes. Rep-PCR primers complement these repetitive sequences and allow for specific binding providing reproducible, unique rep-PCR DNA fingerprint patterns.

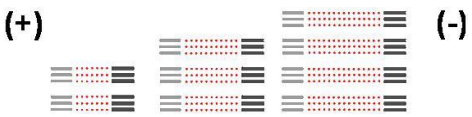
- Rep-PCR primers bind to many specific repetitive sequences interspersed throughout the genome.



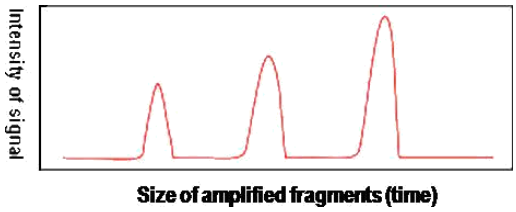
- Multiple Fragments of various lengths are amplified



- Fragments can then be separated by mass and charge via electrophoresis



- A unique rep-PCR DNA fingerprint profile are created with multiple bands of varying intensity



The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of *Escherichia coli* and *Salmonella*. The family of REP elements is generally between 33 and 40 bp in length, has 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* or *Salmonella*. The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi. The application of rep-PCR has been found to be a discriminatory and reproducible tool for microbial typing and for study of molecular epidemiology of pathogens (Versalovic *et al.*, 1998).

Versalovic *et al*. (1991) first described the method of fingerprinting for bacterial genomes based on strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern *et al.*, 1984). REP sequences have been described for numerous enteric bacteria. These dispersed elements are highly conserved within species.

The enterobacterial repetitive intergenic consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements which contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome (Hulton *et al.*, 1991). They have been defined primarily based on sequence data obtained from *E. coli* and *Salmonella typhimurium*.

Rep-PCR can be performed with DNA extracted from bacterial colonies or by a modified method using unprocessed whole cells. REP or ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. ERIC patterns are generally less complex than REP patterns, but both give good discrimination at the strain level. Application of both REP and ERIC PCR to samples to be typed increases the discriminatory power over that of either technique used alone (Olive and Bean, 1999).

Rep-PCR is fast becoming the most widely used method of DNA typing. It shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting. It has been adapted to an automated format in which fluorescently labeled primers are used to create either the REP or ERIC profile and the amplified sequences are separated via a fluorescence-based DNA sequencer (Versalovic *et al.*, 1995).

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Extraction of Bacterial Genomic DNA

(HOT COLD LYSIS METHOD)

Materials Required:

1. Bacterial Culture
2. Hot-water Bath
3. Glassware and Plastic ware
4. TE Buffer (Tris EDTA)
5. Bacteriological Media (LB Broth)

Reagent Preparation:

TE Buffer:

10 mM Tris HCL (pH- 8)
1 mM EDTA

Protocol:

1. Two to three pure and isolated bacterial colonies are to be inoculated in to about 5 ml of enrichment broth (Luria Bertani broth) and then incubate overnight at 37°C.

2. Transfer 1 ml of culture to a 1.5 ml sterile micro-centrifuge tube.

3. Centrifuge at 10,000 rpm for 5 mins and decant the supernatant.

4. Re-suspended the pellet in 50 µl of 1X Tris EDTA (TE) buffer.

5. Boil the suspension in a water bath at 100°C for 10 mins by placing the micro-centrifuge tube in a floating rack.

6. After boiling, place the micro-centrifuge tube immediately into ice for 10mins.

7. Thaw the content and centrifuge at 12,000 rpm for 10 mins.

8. Collect the clear supernatant fluid containing the bacterial genomic DNA in a clean micro centrifuge tube without disturbing the pellet.

9. Store at -20°C.

DNA Quality Confirmation:

1. Prepare a 1.5% solution of Agarose by melting 1.5g of Agarose in 100 ml of 1X TAE (Tris base, acetic acid and EDTA) buffer in a microwave for about 2 mins.
2. Allow to cool for a couple of minutes, then add 0.5µl Ethidium Bromide, stir to mix.
3. Cast a gel using a supplied tray and comb. Allow the gel to set.
4. Load the samples in separate wells and run for 30-60 mins at 80V.

The gel can be viewed in UV gel documentation system.

Materials Required:

1. Thermo cycler
2. Micropipette
3. PCR Master mix (Taq polymerase, dNTP, $MgCl_2$, Reaction Buffer)
4. Primer
5. Template DNA
6. Nuclease free Water
7. Plastic wares

Reaction Mixture for Simplex PCR:	Amount
Template DNA	1.00 µl
$MgCl_2$(25mM)	1µl
dNTP(10mM)	1µl
Taq Polymerase	0.5µl
PCR Buffer (10X)	2.5µl
Forward Primer (10 pM/µl)	0.5 µl
Reverse primer (10 pM/µl)	0.5 µl
Nuclease free water	18.00 µl
Total	**25.00** µl

Cycling conditions (Varies with Primer):

Steps	Temperature	Time	Hold (No. of cycle)
Initial denaturation	95°C	5 mins	1
Denaturation	95°C	30 secs	30
Annealing	56°C	30 secs	
Extension	72°C	30 secs	
Final Extension	72°C	5 mins	1

Agarose Gel Electrophoresis

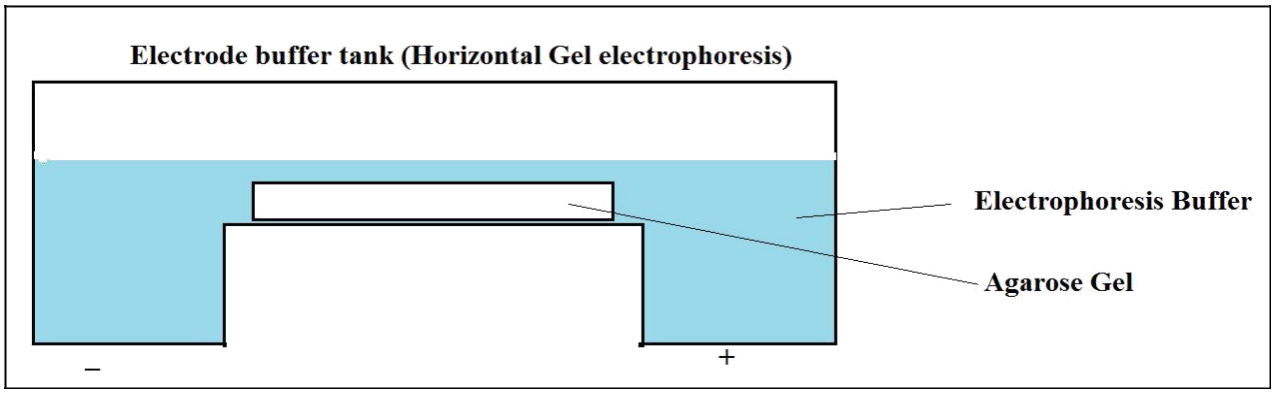
The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and therefore, at any given pH, exist in solution as electrically charged species either as cation or anions. Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. The equipment required for electrophoresis consists basically of two items, as power pack and an electrophoresis unit.

For the majority of DNA samples, electrophoretic separation is carried out in Agarose gels. This is because the DNA molecules and their fragments that are analyzed routinely are considerably larger than proteins and therefore, because most DNA fragments would be unable to enter a polyacrylamide gel, the larger pore size of an agarose gel is required. For example, the commonly used plasmid pBR322 has a molecular weight of 2.4×10^6. However, rather than use such large numbers it more convenient to refer to DNA size in terms of the number of base pairs. So, the plasmid pBR322 has the size of 4.36 kb (4360 base pairs).

As the DNA molecules are negatively charged at neutral and alkaline pH, under the electric field, the DNA molecules would be moving towards the anode. The separation in Agarose gels is achieved due to resistance to their movement caused by the gel matrix. The largest molecules will have the most difficulty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of the DNA molecules during gel electrophoresis will depend on the size, the smallest molecules moving fastest. This is analogous to the separation of proteins in SDS.

Once the gel has been run, the DNA in the gels needs to be stained and visualized. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in solution of ethidium bromide (0.5µg per ml) and then viewed under ultraviolet light (300 nm wavelength)*. The ethidium bromide is a cyclic planer molecule that bind between stacked base-pairs of DNA (i.e. it intercalates). The ethidium bromide concentration therefore buildup at the site of DNA bands and under ultraviolet light the DNA bands fluoresce orange-red. Al little as 10 ng of DNA can be visualized as a 1 cm wide band.

* *Generally the ethidium bromide is added into the gel during its preparation. The concentration of ethidium bromide is used @ 0.5µg per ml of gel.*



1. Preparation of buffers for Agarose Gel Electrophoresis

Material & Reagents: Tris-base, glacial acetic acid, EDTA, Boric acid, Bromo phenol blue, sucrose, Ethidium bromide

Stock solution:

Electrophoresis buffer: For agarose gel electrophoresis, either 1×TAE or 0.5×TBE can be used. For better resolution of DNA bands TAE is preferred to TBE. However, for routine gel runs TBE is preferred due to its long keeping quality. The stock solutions of TAE and TBE are 50×TAE and 10×TBE.

A. Tris Acetate EDTA (TAE) 50X buffer:

Tris base	24.2 g
Glacial acetic acid	5.71 ml
0.5M EDTA (pH 8.0)	10 ml
Make the volume up to 100 ml with double distilled water. Autoclave and store at RT.	

B. Tris Borate EDTA (TBE) 10X buffer:

Tris base	10.8 g
Boric acid	5.5 g
0.5M EDTA	4 ml (pH 8.0)
Make the volume up to 100 ml with double distilled water. Autoclave and store at RT	

C. 6X Gel loading buffer:

Bromophenol blue	: 0.25%
xylene cyanol	: 0.25%
Sucrose	: 40%

Store at 4°C.

D. Ethidium bromide solution (10 mg/ml) (Stock solution)

Ethidium Bromide	: 10 mg
Triple glass distilled water	: upto 1 ml

Working solution of EtBr is 1 mg per ml that is obtained by diluting the stock solution ten times.

Procedure for the Agarose gel electrophoresis:

1. Preparation of Agarose gel (0.8%) 100 ml:
   a. Weigh 0.8 g of Agarose gel base and pour it into the 250 ml flat bottom flask.
   b. Add 1XTAE upto 100 ml.
   c. Heat the suspension with continuous stirring till a clear solution is obtained.
   d. Add working solution of EtBr (0.5µg per ml gel) when the gel solution has the temperature of 60°C and mix the gel solution by stirring.
   e. Caste the gel on the horizontal gel electrophoresis casting unit with the comb placed in it.
   f. Mix the DNA solution in 6X loading dye in the ratio of 2µl of dye with 10 µl of DNA solution.
   g. Load the dye-mixed DNA solution into the well of gel.
   h. Run the electrophoresis @ 5 volts per cm.
2. Viewing the DNA bands.
   a. The DNA bands can be visualized by placing the gel in U.V transilluminator.

Restriction Fragment Length polymorphism (RFLP)

(Modified Sambrook and Russel (Molecular cloning, 3rd edition)

1. Extract the genomic DNA and do the necessary PCR amplification steps to amplify the gene of interest.

2. Take a fresh PCR tube, place it on ice and add 6.5µl of amplified product, 4.0µl of buffer solution (supplied with the enzyme) and 1.5µl of *Sma*I enzyme (the amount is varied as per the DNA concentration and supplied enzyme concentration).

3. Mix them properly by short spinning and immediately place them at 30° C for 5 hours for digestion.

4. Inactivation of enzyme is done at 65°C for 20 minutes (optional as per different enzyme).

5. The RFLP pattern can be visualized by running the digested product in Agarose gel or Polyacrylamide gel electrophoresis.

Isolation of Mammalian DNA

Modified Sambrook and Russel (Molecular cloning, 3rd edition) method

Materials Required:

1. Freshly drawn blood.
2. Cell lysis buffer : 10 mM Tris-cl (pH 8),1mM EDTA (pH 8),0.1%(w/v) SDS (chilled)
3. Ethanol (chilled)
4. Isopropanol (chilled)
5. Potassium acetate solution: 60 ml of 5 M potassium acetate,11.5 ml of glacial acetic acid,28.5 ml H_2O.
6. RBC lysis buffer: 20mM Tris-cl(pH7.6)
7. TE (pH 7.6)
8. DNase free RNase (4mg/ml)
9. Proteinase K (20 mg/ml)

Protocol:

1. Preparation of whole blood for genomic DNA isolation.

2. Transfer 300µl of whole blood in a microcentrifuge tube and add 900µl of RBC lysis buffer. Mix the content properly and incubate it for 10 min at room temperature.

3. Centrifuge the tube at maximum speed for 1 min at room temperature.

4. Discard the supernatant leaving around 20µl at the bottom.

5. Resuspend the pellet in the left out supernatant.

6. Add 600µl of ice cold cell lysis buffer and mix the content properly.

7. Add 3µl of Proteinase K solution and incubate at 55^0C for 1 hr.

8. Allow the reaction to come down to room temperature and add 3µl of 4 mg/ml DNase free RNase. Incubate at 37^0 C for 20 min.
9. Allow the sample to cool to room temperature and add 200 µl of potassium acetate solution. Mix the contents by inverting the tube repeatedly for about 20 sec.

10. Pellet the precipitated protein/SDS complex by centrifugation at maximum speed for 3 min at 4°C.
11. Transfer the supernatant to a fresh microcentrifuge tube containing 600µl of chilled Isopropanol. Mix the solution well and then recover the DNA by centrifugation at maximum speed for 1 min at room temperature.

12. Remove the supernatant and add 600 µl of chilled 70 % ethanol to the DNA pellet. Invert the tube several times and centrifuge the tube at maximum speed for 1 min at room temperature.

13. Carefully remove the supernatant and allow the DNA pellet to dry in dry bath.

14. Redissolve the pellet of DNA in 100µl of TE buffer or Nuclease free water (NFW).

RNA isolation

(**TRIzol Method**)

Materials Required:

1. Trizol Reagent (Amresco/Invitrogen)
2. Diethylpyrocarbonate (DEPC) (Amresco/Himedia)
3. Plastic ware and glassware

Protocol:

1. Take 250 µl of whole blood in a 1.5 ml microcentrifuge tube and add 750 µl TRIzol reagent. Mix vigorously by vortexing.

2. Add 200 µl of chloroform to the homogenized lysate and vortex; keep at room temperature for 2-15 minutes.

3. Centrifuge at 12,000 g for 15 min and transfer the aqueous phase on the top to a fresh microcentrifuge tube.

4. Add an equal volume of isopropanol and mix by vortexing. Keep at room temperature for 10 min to precipitate the RNA.

5. Centrifuge at 12,000 g for 10 min at 2-8 °C to pellet the precipitated RNA.

6. Discard carefully without disturbing the pellet.

7. The RNA pellet is washed using 70% ethanol by centrifugation at 7500 g for 5 min.

8. The RNA pellet is dried and the RNA is dissolved in 30 µl of nuclease free water by keeping at 55-60 °C for 10 min.

Material Required:

1. Total RNA
2. Random Hexamer (50 ng/µl)
3. Reverse transcriptase (200 U/µl)
4. dNTP (10 mM)
5. RNase inhibitor (40 U/µl)
6. 5X RT Buffer
7. Nuclease free water
8. Nuclease free plastic ware

Protocol:

A. Reverse transcription (cDNA synthesis)

1. cDNA synthesis by using the following reaction condition:

Total RNA	3 µl
Random Hexamer	0.5 µl
Nuclease free water	3 µl

2. Mix the contents properly and spin briefly. Apply the following reaction condition:

70°C	5 Min
25°C	10 Min
4°C	Hold

3. Add the following reagents

5X RT buffer	2 µl
RNase inhibitor	0.5 µl
10 mM dNTP Mix	0.5 µl
Reverse transcriptase	0.5 µl

4. Mix properly, spin it and place in the thermal cycler with following conditions:

25°C	5 Min (Primer annealing)
42°C	1 Hr (Extension)
70°C	10 Min (Inactivation of enzyme)
4°C	Hold

The cDNA thus formed can be used for downstream processing or stored at -20° C for future use.

Repetitive Element based (REP) PCR

Requirements:

1. Thermal cycler
2. Micropipette
3. Master mix
4. Primer
5. Template DNA
6. $MgCl_2$
7. Nuclease Free Water
8. Plasticwares

DNA Extraction: Hot-Cold lysis Method

Reaction mixture for REP-PCR:

Master Mix	12.5 µl
Primer	2.0 µl (25picomol)
Template DNA	3.0 µl
Nuclease Free Water	7.0 µl
$MgCl_2$	0.5µl
Total =	**25.0 µl**

Cycling condition:

Steps	Temperature	Time	Hold (No. of cycle)
Initial Denaturation	95^0C	5 min	1
Denaturation	90^0C	30 sec	35
Annealing	50^0C	30 sec	
Annealing	52^0C	1 min	
Extension	72^0C	1 min	
Final Extension	72^0C	8 min	1
Hold	4^0C	Infinite time	

Gel Electrophoresis using 1.8% Agarose gel at 80V for 90 mins.

Materials required:

1. Pipettes.
2. Plastic wares including microfuge tubes (1.5 and 2.0 ml), and tips.
3. Restriction enzyme *Xba I* along with Tango buffer.
4. PFGE grade agarose (Bio-Rad).
5. Electric fine balance.
6. Spectrophotometer.
7. Microcentrifuge apparatus.
8. Low melting point agarose.
9. Plug molds.
10. PFGE Chef Mapper.
11. Gel Documentation System.
12. Thermomixer.
13. Microwave oven.
14. Fine spatulas.

Preparation of gel plugs from broth culture:

1. Inoculate test cultures on Luria agar plates; streak for isolated colonies. Incubate at 37°C.

2. Inoculate isolated colony from agar plate into 4-5 ml L-broth.

3. Incubate broth on a shaker (100-150 rpm) for 16-18 h at 37°C.

(**Note:** Broth cultures can be incubated without shaking if equipment is not available. Do not incubate broth longer than 16-18 h. If cultures cannot be processed early in the morning, refrigerate cultures or keep them on ice.)

4. Inoculate 1 ml of the overnight culture to 8 ml of LB broth

5. Incubate at 37°C with vigorous shaking until the OD_{6oo} reaches at 0.8 to 1.0 (Usually it takes 3 hr time). At routine intervals, take out 1 ml portion of the broth aseptically and check OD_{6oo} value.

6. Label two sets of 1.5-ml microcentrifuge tubes with strain numbers. Transfer 1.0 ml of broth culture to labeled 1.5-ml microcentrifuge tubes. Refrigerate or keep remainder of cell suspensions on ice until the plugs are made and lysis started.

7. Centrifuge 5 min at 1000 x g at 4°C to get packed cells.

8. Carefully remove supernatant with pipette.

9. Carefully wash cells in 500 µl of cell suspension buffer and resuspending them in 500 µl of cell suspension buffer.

OR

Directly mix cultures from LB agar plate in 500µl of cell suspension buffer

10. Incubate for 10 min at 50°C.
11. Prepare 2 % of low melting point agarose in a microwave oven and store it at 50°C for l0 min in a heating block.
12. Mix the cell suspension with equal volume (500 ul) of molten (2%) low melting agarose at 50°C.
13. Label the wells of PFGE plug molds with culture number. Dispense part of the mixture into appropriate well(s) in the plug mold. Do not allow bubbles to form. Allow plugs to solidify at room temperature for 1 h or 15 min at 4^{O}C. Use duplicate wells to prepare at least two plugs for each culture.
14. Push the gel plug using the bar into 2 ml tube; add 500 µl of lysozyme solution

(**Note:** Lysozyme solution should be prepared freshly on the day of use.)

15. Soak the plug in lysozyme solution at 37°C for at least 2 h (plugs can be kept overnight with lysozyme solution)
16. Carefully remove lysozyme solution and rinse the plugs two times with reagent grade water at the interval of 30 min each.
17. Immerse the plug with 500 µl of proteinase K solution and incubate in overnight at 50^{O}C.
18. Wash the plugs with l.0X washing buffer two times with agitation at room temperature for an hour each.

(**Note:** Second washing should be carried out with washing buffer containing 1 mM phenylmethyl sulphonyl fluride (PMSF).

19. Wash twice with 0.1 X washing buffer for 30 min at room temperature.

(**Note:** Plugs can be stored at least for 2 months at 4°C in 1.0X washing buffer. However, additional wash with 0.1X washing buffer is required' to proceed to next step.)

Restriction digestion of DNA embedded in gel plugs:

1. Carefully remove the washing buffer and add 200-500 ul of 1.0X Tango buffer and incubate the plugs for 1 h at room temperature.
2. Carefully remove the buffer and add fresh 200-300 ul of 1.0X Tango buffer.
3. Add 40 units of Xba I restriction enzyme, mix gently, and incubate at 37°C in a water bath or thermomixer for overnight.

Preparation of gel and electrophoresis unit for PFGE:

1. Make 2-2.2 L of 0.5X Tris-Borate EDTA Buffer (TBE)

2. Make 1% Pulse Field Certified (PFC) Agarose in 0.5X TBE.

(**Note:** Confirm that casting platform is leveled before pouring gel, that front of comb holder and teeth should face towards bottom of gel, and that height of comb is 2 mm above the surface of the gel platform so wells will be right size for inserting the plugs.)

3. Cool molten PFC agarose in 65°C water bath for 5-6 min.

4. Prepare the gel plugs as per the size of the comb. Put gel plugs on the comb one by one and then carefully pour agarose into gel casting tray fitted with comb. Be sure there are no bubbles. Allow gel to solidify for an hour at room temperature.

5. Keep casting frame or lower corner posts in chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of the unit.

6. Turn on cooling module (14°C), power supply, and pump (setting speed at 60-70) approximately 30 min to 1 h before gel is to be run.

7. Remove comb from gel (gel should harden for 1h before being used for the electrophoresis).

Instructions to run the machine: Steps

Select following on the CHEF Mapper

1. Press Auto Algorithm.
2. After Molecular, weight: Low, key in 30, press K-BASES, press ENTER
3. After Molecular weight: High, key in700, press K-BASES, press ENTER.
4. The lower line will display Calibration factor [ ], key 1.5 press ENTER. The lower line displays the physical parameters for the run: O.5X TBE, 14°C, 1% PFC agarose. Press ENTER
5. The screen display shows the calculated electrical parameters: 6 V/cm, Run time 16 h. Included angle 120°C. Press ENTER.
6. The electrical parameters continue on the next screen: Int Sw.Tm 2.16sec, Fin. Sw. Tm. 63.8 sec
7. Ramping factor: a [linear]. Press ENTER
8. The final display is A program is In memory. Start the run by pressing START RUN:

9. When electrophoresis run is over, turn off equipment and remove the gel.
10. Stain the gel with ethidium bromide for 30 min in a covered container.
11. Destain the gel for 15-20 min.
12. Capture image on Gel Doc.
13. Drain buffer from electrophoresis chamber. Rinse chamber with 2L of reagent grade water, drain and dry the instrument.

Preparation of buffers and media

1. 1.0 M Tris-HCl (PH:7.2)

2. 0.5 M EDTA (PH:8.0)

3. Luria Burtoni broth.

4. Luria Burtoni agar plates.

5. 5.0 M NaCl.

6. 100 mM PMSF.(Store in -20^{o}C)

7. Cell suspension buffer (10ml)	Addition
10.0 mM Tris-HCl (PH: 7.2)	0.1 ml of 1.0 M Tris-HCl, PH: 7.2
20.0 mM NaCl	0.04 ml of 5.0 M NaCl.
50.0 mM EDTA	1.0 ml of.5M EDTA (PH:8.0)

Adjust the volume with sterile reagent grade water to 10 ml.

8. Lysozyme solution (50ml)	Addition
1mg/ml Lysozyme in	50.0 mg Lysozyme.
10.0 mM Tris-HCl (PH: 7.2)	0.5 ml of 1.0 M Tris-HCl, PH: 7.2
50.0 mM NaCl	0.5 ml of 5.0 M NaCl.
0.2% Na- deoxycholate	0.1g of Na- deoxycholate.
0.5% Na-laurylsarcosine	0.25g of Na-laurylsarcosine

Adjust the volume with sterile reagent grade water to 50 ml.

9. Proteinase K solution (50ml)	Addition
1.0 mg/ml Proteinase K	50.0 mg Proteinase K
100 mM EDTA ((PH: 8.0)	10.0 ml of 0.5M EDTA, PH: 8.0
0.2% Na- deoxycholate	0.1g of Na- deoxycholate.
1.0% Na-laurylsarcosine	0.5g of Na-laurylsarcosine

Adjust the volume with sterile reagent grade water to 50 ml.

10. Washing buffer 1X (50 ml)	Addition
20.0 mM Tris-HCl (PH: 8.0)	1.0 ml of 1.0 M Tris-HCl, PH: 7.2
50.0 mM EDTA	5.0 ml of 0.5 M EDTA, PH: 8.0

Adjust the volume with sterile reagent grade water to 50 ml.

11. Washing buffer 1X with PMSF (15 ml)	Addition
20.0 mM Tris-HCl (PH: 8.0)	0.3 ml of 1.0 M Tris-HCl,PH: 7.2
50.0 mM EDTA	1.5 ml of 0.5 M EDTA, PH: 8.0
1.0 mM PMSF	0.15 ml of 100 mM PMSF

Adjust the volume with sterile reagent grade water to 15 ml.

Materials required:

1. Thermalcycler
2. Micropipette
3. REP-PCR master mix
4. 10X PCR buffer
5. Primer
6. Template DNA
7. *Taq* DNA Polymerase
8. Plasticwares

Reaction mixture for REP-PCR:

REP-PCR Master mix	18.0 µl
10X PCR buffer	2.5 µl
Primer	2.0 µl
Taq DNA Polymerasre	0.5 µl
Template DNA	2.0 µl (50ng/ul)
Total	**25.0 µl**

Cycling condition:

Steps	Temperature	Time	Hold (No. of cycle)
Initial Denaturation	94^0C	2 min	1
Denaturation	94^0C	30 sec	35
Annealing	45^0C	30 sec	
Extension	70^0C	1.30 min	
Final Extension	70^0C	3 min	1
Hold	4^0C	Infinite time	

DiversiLab chip loading:

Protocol:

1. Briefly vortex and spin ladder and marker.
2. Load 9 µl of gel-dye mix in chip priming well. (black circle)
3. Pressurize chip for 2 min starting syringe 1 ml.
4. Load 9 µl of gel-dye mix in the remaining G wells.
5. Load 5 Load 1 µl of of marker in each well, except the G wells.
6. Load 1 µl of ladder in the specified well.
7. Load 1 µl of PCR product in assigned sample well.
8. Vortex chip for 3 min.

Preparation of gel-dye mix:

1. Allow the DNA gel matrix and DNA dye concentrate to warm to room temperature.
2. Vortex and spin the gel matrix and dye concentrate. Briefly spin down.
3. Add 200 µl of DNA gel matrix and 10 µl of DNA dye concentrate
4. Briefly vortex the tube until the solution is homogenous.
5. Transfer the solution to a spin filter tube.
6. Centrifuge the tube at 1,500×g for 10 min at room temperature.
7. Store the gel-dye mix at 4^0C.

1. Grow the bacterial culture in broth or petriplate or slant.
2. Isolate the genomic DNA using proper protocol OR good DNA isolation kit. It is recommended to have at least 150 - 200 ng of pure DNA without any contaminant like protein, lipid, media, etc.
3. The completely pure DNA is used for PCR process using standardized primer at desired annealing temperature.
4. After PCR process is over, the amplified product is run in agarose gel under standardized condition and visualized under UV light.

PCR master mix components

1. Template genomic DNA	3.0 µl
2. Primer (18pico mol)	2.0 µl
3. $MgCl_2$	1.0 µl
4. Taq polymerase (2U ml^{-1})	1.0 µl
5. 10X PCR buffer	3.0 µl
6. dNTPs	0.5 µl
7. Nuclease free water	14.5µl
Total	25 µl

PCR running conditions

Heated lid on	105 ^{0}C		
Initial denaturation	94 ^{0}C	5 minutes	1 cycle
Denaturation	94 ^{0}C	1 minute	40 cycles
Annealing	34 ^{0}C	1 minute	
Extention	72 ^{0}C	2 minutes	
Final extention	72 ^{0}C	10minute	1 cycle
Final halt	0 ^{0}C – 4 ^{0}C	forever	

Gel running condition

Electricity	80 volts
Time	80 minutes
Gel percentage	1.5 %

CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB) METHOD:

Materials Required:

1. Plant leaves
2. CTAB buffer
3. Chloroform: Isoamyl Alcohol (24:1)
4. 7.5 M Ammonium acetate
5. Absolute Ethanol (ice cold)
6. 70% Ethanol (ice cold)
7. Agarose
8. Ethidium Bromide Solution (EtBr)
9. Mortar and Pestle
10. Glassware and Plastic wares
11. TE Buffer
12. Tween 20

Reagent Preparation:

CTAB Buffer (For 100ml):

2.0g CTAB
10.0ml of 1M Tris (pH 8.0)
4.0 ml of 0.5M EDTA (pH 8.0)
28.0 ml of 5ml NaCl
1g Polyvinylpyrrolidone (PVP)

- Autoclave Tris, EDTA and NaCl without CTAB and PVP.
- Adjust all to pH 5.0 with HCl and make up with 100ml with H_2O.

PROTOCOL:

1. Wash 1g of plant tissue with Tween 20.
2. Grind the tissue in pestle and mortar by adding approximately 500µl of CTAB buffer.
3. Transfer the CTAB/plant extract mixture to a microcentrifuge tube.
4. Incubate the CTAB/plant extract mixture for about 15mins at 55^{o}C in water bath/thermomixer.
5. After incubation spin the CTAB/plant extract mixture at 8000 rpm for 5 mins to spin down cell debris.
6. Transfer the supernatant to clean microcentrifuge tubes.
7. To each tube add 250 µl of Chloroform: Isoamyl alcohol (24:1) and mix the solution by inversion. After mixing spin the tubes at 13000 rpm for 5 mins.
8. Transfer the upper aqueous phase only to a clean microcentrifuge tube.
9. To each tube add 50 µl of 7.5M Ammonium acetate followed by 500µl of ice cold absolute ethanol.
10. Invert the tubes slowly several times and incubate for 1hr at -20^{o}C after the addition of ethanol to precipitate the DNA.
11. Spin at 10,000 rpm for 10 minutes. Discard the supernatant and to the pellet add 500µl of ice cold 70% ethanol and again centrifuge at 10,000 rpm for 5mins.
12. Discard the ethanol (repeat the above step).
13. Dry the pellet (approx 15 mins, do not over dry).
14. Dissolve in 100 µl TE buffer.
15. Allow a short spin.
16. Agarose gel electrophoresis of the DNA will show integrity of the DNA.

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