Faculty of Agriculture **Benha University**

Genetics and Genetic Engineering Department

Postgraduate

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Answer model

Course: genetic technique

The answer of the first question

1- Write in concise manner about types of molecular markers

- **Markers as tools in genetic studies**
  - Types of marker: isoenzymes, RFLPs, RAPDs, SCARs, SSRs, AFLPs, SNPs. Discovery, genetic interpretation, properties, methodology, comparison of types of markers
  - Linkage analysis with markers. Genetic mapping. Use of computer programs for mapping
  - Characterization of genetic variability with molecular markers and applications. Basic concepts and applications on linkage disequilibrium and association mapping

- **Marker-assisted selection**
  - Use of markers irrespective of their position in the genome. Seed quality assessment, cultivar identification, pedigree analysis and other applications
  - Location of markers linked to major genes. Methods of major gene selection with markers. Introgression of genes from other species. Whole genome selection in backcross programmes. Genomic selection.

- Dissection, characterization and selection of quantitative traits with molecular markers

- **Genome analysis and applications**
  - Comparative mapping and genomics. Colinearity between genomes at the genus, family and plant kingdom levels and applications
  - Sequenced models and crop plants. Uses of EST collections and microarrays. Candidate gene strategies. Positional cloning. Mutant collections: TILLING
  - Molecular genetics of disease resistance and fruit quality. Applications for the selection of these characters in a breeding programme

2- Compare between negative and positive marker

- **Positive** selectable markers are selectable markers that confer selective advantage to the host organism. An example would be antibiotic resistance, which allows the host organism to survive antibiotic selection.
- **Negative** selectable markers are selectable markers that eliminate or inhibit growth of the host organism upon selection. An example would be thymidine kinase, which makes the host sensitive to ganciclovir selection.

3- Explain the important properties of ideal markers for MAS

- Easy recognition of all possible phenotypes (homo- and heterozygotes) from all different alleles
- Demonstrates measurable differences in expression between trait types or gene of interest alleles, early in the development of the organism

- Testing for the marker does not have variable success depending on the allele at the marker locus or the allele at the target locus (the gene of interest that determines the trait of interest).
- Low or null interaction among the markers allowing the use of many at the same time in a segregating population
- Abundant in number
- Polymorphic

4- How to join between phenotypic trait and marker on DNA level

Morphological markers are associated with several general deficits that reduce their usefulness including:

- the delay of marker expression until late into the development of the organism
- dominance
- deleterious effects
- pleiotropy
- confounding effects of genes unrelated to the gene or trait of interest but which also affect the morphological marker (epistasis)
- rare polymorphism
- frequent confounding effects of environmental factors which affect the morphological characteristics of the organism

To avoid problems specific to morphological markers, the DNA-based markers have been developed. They are highly polymorphic, exhibit simple inheritance (often codomimant), are abundant throughout the genome, are easy and fast to detect, exhibit minimum pleiotropic effects, and detection is not dependent on the developmental stage of the organism. Numerous markers have been mapped to different chromosomes in several crops including rice, wheat, maize, soybean and several others, and in livestock such as cattle, pigs and chickens. Those markers have been used in diversity analysis, parentage detection, DNA

fingerprinting, and prediction of hybrid performance. Molecular markers are useful in indirect selection processes, enabling manual selection of individuals for further propagation.

'Major genes' that is responsible for economically important characteristics are frequent in the plant kingdom. Such characteristics include disease resistance, male sterility,[7] self-incompatibility, and others related to shape, color, and architecture of whole plants and are often of mono- or oligogenic in nature. The marker loci that are tightly linked to major genes can be used for selection and are sometimes more efficient than direct selection for the target gene. Such advantages in efficiency may be due for example, to higher expression of the marker mRNA in such cases that the marker is actually a gene. Alternatively, in such cases that the target gene of interest differs between two alleles by a difficult-to-detect single nucleotide polymorphism, an external marker (be it another gene or a polymorphism that is easier to detect, such as a short tandem repeat) may present as the most realistic option. There are several indications for the use of molecular markers in the selection of a genetic trait.

In such situations that:

- the selected character is expressed late in plant development, like fruit and flower features or adult characters with a juvenile period (so that it is not necessary to wait for the organism to become fully developed before arrangements can be made for propagation)
- the expression of the target gene is recessive (so that individuals which are heterozygous positive for the recessive allele can be crossed to produce some homozygous offspring with the desired trait)
- there is requirement for the presence of special conditions in order to invoke expression of the target gene(s), as in the case of breeding for disease and pest resistance (where inoculation with the disease or subjection to pests would otherwise be required). This advantage derives from the errors due to unreliable inoculation methods and the fact that field inoculation with the pathogen is not allowed in many areas for safety reasons. Moreover, problems in the recognition of the environmentally unstable genes can be eluded.

- the phenotype is affected by two or more unlinked genes (epistatis). For example, selection for multiple genes which provide resistance against diseases or insect pests forgene pyramiding.

The cost of genotyping (an example of a molecular marker assay) is reducing while the cost of phenotyping is increasing[*citation needed*] particularly in developed countries thus increasing the attractiveness of MAS as the development of the technology continues.

Generally the first step is to map the gene or quantitative trait locus (QTL) of interest first by using different techniques and then using this information for marker assisted selection. Generally, the markers to be used should be close to gene of interest (<5 recombination unit or cM) in order to ensure that only minor fraction of the selected individuals will be recombinants. Generally, not only a single marker but rather two markers are used in order to reduce the chances of an error due to homologous recombination. For example, if two flanking markers are used at same time with an interval between them of approximately 20cM, there is higher probability (99%) for recovery of the target gene.

5- Write the steps of isolation and purification of genomic DNA from plant

, animal and bacteria respectively

Plant DNA Extraction Protocol

Source: Protocol modified from Keb-Llanes et al. (2002) Plant Molecular Biology Reporter, 20: 299a–299e.

Introduction

Plant materials are among the most difficult for high quality DNA extractions. The key is to properly prepare the tissues for extraction. In most cases this involves the use of liquid nitrogen flash freezing followed by grinding the frozen tissue with a mortar and pestle. Liquid nitrogen is difficult to handle and it is dangerous in an open laboratory environment such as a classroom. For this reason we have modified a very simple plant DNA extraction protocol to use fresh tissue. We have

also used tissue prepared in advance by dessication. The protocols and results are presented here.

Reagents and Buffers

Extraction Buffer A (EBA) Per 100 mL

2% (w/v) hexadecyltrimethylammonium bromide (CTAB) 2.0 g
100 mM Tris (pH 8.0) (Use 1 M stock) 10 mL
20 mM EDTA (Use 0.5 M stock) 1 mL
1.4 M NaCl 8.2 g
4% (w/v) polyvinylpyrrolidone (PVP) 4.0 g
0.1% (w/v) ascorbic acid 0.1 g
10 mM β-mercaptoethanol (BME)* (Use 14.3 M stock) 70 µL

Extraction Buffer B (EBB) Per 100 mL

100 mM Tris-HCl (pH 8.0) (Use 1 M stock) 10 mL
50 mM EDTA (Use 0.5 M stock) 2.5 mL
100 mM NaCl 0.6 g
10 mM β-mercaptoethanol (BME)* (Use 14.3 M stock) 70 µL

TE Buffer Per 100 mL

10 mM Tris (pH 8.0) (Use 1 M stock) 1.0 mL
1 mM EDTA (Use 0.5 M stock) 50 µL

Other Required Reagents

20% (w/v) sodium dodecyl sulphate (SDS)
5 M potassium acetate (Stored at –20oC)
3 M sodium acetate (pH 5.2)
70% ethanol (stored at -20oC)
Absolute isopropanol (stored at -20oC)

Extraction Protocol

1. Weight out 0.3 g of plant tissue

2. Place tissue on a clean glass slide. Chop the tissue into a paste using a clean single edge razor blade. (we have also modified a Dremel Roto-tool for use as a simple tissue homogenizer with good success)

3. Immediately transfer tissue to a 1.5 mL microcentrifuge tube (use Kontes #749520-0090) and (optional) further grind tissue with a tube pestle (Kontes #749521-1590)

4. Once the sample is prepared add 300 µL EBA, 900µl EBB, and 100 µl SDS.

5. Vortex and incubate at 65oC for 10 min.

6. Place tube on ice and add 410 µL cold potassium acetate. Mix by inversion and place tube back on ice for 3 min.

7. Centrifuge at 13,200 rpm for 15 min. (If possible, use a refrigerated micro-centrifuge set to 4oC)

8. Transfer 1 mL of the supernatant to a new 1.5 mL microcentrifuge tube, add 540 µL of ice cold absolute isopropanol, and incubate in ice for 20 min.

9. Centrifuge at 10,200 rpm for 10 min. discard the supernatant. Wash the pellet once in 500 µL 70% ethanol and let dry

10. Resuspend the dry pellet in 600 µL of TE. Add 60 µL 3M sodium acetate (pH 5.2) and 360 µL ice cold absolute isopropanol. Incubate on ice for 20 min.

11. Repeat Steps 9−11 twice.

12. Resuspend the pellet in 50 µL TE and carry out agarose gel QC.

Agarose Gel QC

1. cast a 1.0% (w/v) regular agarose gel in 1X TBE

2. Place 5 µL of extracted DNA and 5 µL sterile water

Genomic DNA

in a 0.2 mL microcentrifuge tube along with 2 µL of

gel tracking dye.
3. Run the gel for 20 min. at 100v.
4. Stain gel and view result.

PCR QC

Obtaining what appears to be good high molecular weight genomic is only the first line of QC for this protocol. The ultimate test is to see if the DNA can be used to amplify a PCR product. The test case used in developing this protocol was leaf tissue from the coleus plant (*Coleus blumei*). In order to test the DNA for PCR amplification the gene encoding tyrosine aminotransferase (GenBank #AJ458993) was submitted for PCR primer selection using the IDT SciTools software PrimerQuest. The software chose the following sequences:
Tat FOR:5'- ATA AAC CCT GGG AAC CCA TGT GGA –3'
Tat REV: 5'- AAC TTT GGG CTC ATC AAA GTG CCG –3'
These sequences were synthesized and a PCR amplification carried out using the conditions; 5 min. 94oC; 35 x (30 sec. 94oC, 30 sec. 57oC; 30 sec 72oC); 7 min. 72oC.

DNA extraction from bacteria

CTAB/ NaCl solution (4.1 g NaCl added to 80 ml of water, 10 g of CTAB is slowly added while heating. The volume is made up to 100 ml).
1. Cultures of bacterial cells were made in Wilbrink (1920) medium at 22°C. 50 ml of liquid culture was centrifuged for 10 minutes. The supernatant was removed
and the pellet washed twice in NE buffer (0.15 M NaCl/ 50 mM EDTA).
2. Pellet was resuspended in 3860 ml of TE (Tris 10 mM; EDTA 1mM) buffer; 40 ml of proteinase K (10 mg/ml) and 100 ml of 20% SDS are added. Mixture was incubated for at least 2 hr to allow complete lysis.
3. 667 ml of 5 M NaCl was added to give a final concentration of 0.7 M.
4. 533 ml of CTAB/NaCl (10% CTAB; 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated at 65°C for 30 min.
5. An equal volume of chlorofrom/isoamyl alcohol (24:1) was added and the tube was centrifuged for 15 minutes.

6. The aqueous phase was removed into a clean 15-ml tube and an equal volume of
phenol/chloroform/isoamyl alcohol (25:24:1) added. After mixing and centrifuging
the supernatant was removed.
7. 0.6 volume of isopropanol was added to the supernatant to precipitate the nucleic
acids. At this point a white precipitate was clearly visible and could be removed with a sealed pasteur pipette (when the polysaccharides coprecipitate with the nucleic acids a clear, gelatinous mass is obtained).
8. The precipitate was washed by dipping the pasteur pipette in 70 % ethanol. It was then allowed to dissolve in 500 ml of sterile water and the solution was stored at -20 °C.
The volume of TE added in step 2 should be carefully measured by taking into consideration the volume of cells present. This is important in order to add the right
volume of 5 M NaCl to give a final concentration of 0.7 M. When the aqueous phase is viscous and is difficult to pipette after addition of chloroform/isoamyl

6- Summarize the steps of isolation and purification of mRNA from Plant tissue protocol and mention the precautions for this procedure

Total RNA isolation protocol

The procedure is suitable for all types of tissues from wide variety of animal (and blood) and plant species. All steps are performed at weak acid pH (HEPES free acids) and at room temperature (RT) (without ice) and without DEPC-treated water. RNA precipitate with lithium chloride (LiCl) for increased stability of the RNA preparation and improvement of cDNA synthesis. The following protocol is designed for small and large tissue samples (tissue volume 10-200 µl), which normally yield about 10-500 µg of total RNA.
Materials for total RNA isolation

- **GuTC** extraction buffer: 4 M guanidine thiocyanate, 1% N-lauroylsarcosine (Na salt, Sarkosyl), 10 mM EDTA, 50 mM HEPES, pH ~5.3;

- Buffer-saturated phenol, pH 4.5-6.6;
- Chloroform-isoamyl alcohol mix (24:1);
- 100% isopropanol (isopropyl alcohol, 2-propanol);
- 70% ethanol;
- 10 M LiCl;
- Fresh Milli-Q water (or Milli-Q ultrapure BioPak water) or autoclaved 1xTE (0.1 mM EDTA, 10 mM Tris-HCl, pH 7.0). When an ultrafiltration cartridge (BioPak) is utilized at the point-of-use, the water is suitable for genomics applications (quality at least equivalent to DEPC-treated water) and cell culture.

1. 2 ml Eppendorf Safe-Lock microcentrifuge tube with tissue sample and glass ball freeze at -80°C, grind in the MM300 Mixer Mill for 2 min at 30 Hz.
2. In 2 ml tube with mechanically disrupted tissue sample add fresh 1 ml **GuTC** extraction buffer, vortex very well, and incubate the sample for 2 hours or longer at +4°C. Spin at maximum speed on table microcentrifuge for 10 minutes at +4°C.
3. Transfer 0.8 ml of the supernatant (the pellet contains polysaccharides and high molecular weight DNA) to a fresh tube with an equal volume of buffer-saturated phenol, vortex and incubate for 5 minutes at room temperature. Add 300 µl of chloroform-isoamyl alcohol, vortex well. Spin at maximum speed on table microcentrifuge for 5 minutes at at +4°C. Repeat this step.
4. Transfer the aqueous phase to a fresh microcentrifuge 2 ml tube with 700 µl of chloroform-isoamyl alcohol, vortex well. Spin at maximum speed on table microcentrifuge for 5 minutes.
5. Transfer the aqueous phase to a fresh microcentrifuge 2 ml tube with an equal volume of 2-propanol, vortex well. Spin at maximum speed on table microcentrifuge at room temperature for 2 minutes. Wash the pellet once with 1.5 ml 70% ethanol. Spin at maximum speed on table microcentrifuge for 5 minutes.
6. Dissolve the pellet (do not dry) in 400 µl 1xTE at 55°C about 10-20 min, with vortex. If the pellet cannot be dissolved completely, remove the debris by

spinning the sample at maximum speed on table microcentrifuge for 5 minutes at room temperature.

7. Transfer the supernatant to a new tube, then add an equal volume of 10 M LiCl and chill the solution at -20°C for several hours. Spin at maximum speed on table microcentrifuge for 10 minutes at +4°C. Carefully remove and discard (or save, Fig.1) supernatant (contains: small RNA < 200 nt and DNA). Wash pellet with 1.5 ml 70% ethanol, vortex well, microcentrifuge, discard the ethanol, don't dry the pellet. Dissolve the pellet in 200-400 µl fresh milliQ water (BioPak) or 1xTE.

Load 5 µl of the solution onto a standard (non-denaturing) 1.5 % agarose gel with 1xTHE buffer to check the amount and integrity of the RNA. Add ethidium bromide (EtBr) to the gel to avoid the additional (potentially RNAse-prone) step of gel staining. Load a known amount of DNA in a neighboring lane to use as standard for determining the RNA concentration. Intact RNA should exhibit sharp band(s) of ribosomal RNA.

Notes

1. There is widespread belief that RNA is very unstable and therefore all the reagents and materials for its handling should be specially treated to remove possible RNAse activity. We have found that purified RNA is rather stable and, ironically, too much anti-RNAse treatment can become a source of problems. This especially applies to DEPC-treating of aqueous solutions, which often leads to RNA preparations that are very stable but completely unsuitable for cDNA synthesis. We have found that simple precautions such as wearing gloves (only for your protection from chemicals), avoiding speech over open tubes, using aerosol-barrier tips, and using fresh 1xTE (or 1xTHE) solution (or Milli-Q ultrapure BioPak water) for all solutions are sufficient to obtain stable RNA preparations.
   When an ultrafiltration cartridge (BioPak) is utilized at the point-of-use, the water is suitable for genomics applications (quality at least equivalent to DEPC-treated water) and cell culture. The BioPak cartridges has been validated in Millipore laboratories to warrant the production of pyrogen-free (less than 0.001 Eu/ml), RNAse-free (less than 0.01 ng/ml) and DNase-free (less than 4 pg/µl) ultrapure water, while maintaining both the

resistivity and total organic carbon (TOC) of the treated water, it replaces the lengthy diethylpyrocarbonate (DEPC) treatment process to remove nucleases from purified water.
All organic liquids (phenol, chloroform and ethanol) can be considered essentially RNAse free by definition, as is the dispersion buffer containing guanidine thiocyanate.

2. The final concentration of guanidine thiocyanate may need to optimized for certain plant tissue from 2 to 4 M.
3. The volume of tissue should not exceed 1/5 of the extractiom buffer volume. To avoid RNA degradation, tissue dispersion should be carried out as quickly and completely as possible, ensuring that cells do not die slowly on their own. To adequately disperse a piece of tissue usually takes 2-3 minutes of triturating using a pipet, taking all or nearly all volume of buffer into the tip each time. The piece being dissolved must go up and down the tip, so it is sometimes helpful to cut the tip to increase the diameter of the opening for larger tissue pieces. Tissue dispersion can be performed at room temperature. The tissue dispersed in extraction buffer produces a highly viscous solution. The viscosity is usually due to genomic DNA. This normally has no effect on the RNA isolation (except for dictating longer periods of spinning at the phenol-chloroform extraction steps), unless the amount of dissolved tissue was indeed too great.
4. Note that isolating genomic DNA and RNA not requires very gentle mixing because the DNA and RNA not be sheared by vortexing.
5. RNA degradation can be assessed using non-denaturing electrophoresis. The first sign of RNA degradation on the non-denaturing gel is a slight smear starting from the rRNA bands and extending to the area of shorter fragments. RNA showing this extent of degradation is still good for further procedures. However, if the downward smearing is so pronounced that the rRNA bands do not have a discernible lower edge, the RNA preparation should be discarded. The amount of RNA can be roughly estimated from the intensity of the rRNA staining by ethidium bromide in the gel, assuming that the dye incorporation efficiency is the same as for DNA (the ribosomal RNA may be considered a double-stranded molecule due to its extensive

secondary structure). The rule for vertebrate rRNA - that in intact total RNA the upper (28S) rRNA band should be twice as intense as the lower (18S) band - does not apply to invertebrates. The overwhelming majority have 28S rRNA with a so-called "hidden break". It is actually a true break right in the middle of the 28S rRNA molecule, which is called hidden because under non-denaturing conditions the rRNA molecule is held in one piece by the hydrogen bonding between its secondary structure elements. The two halves, should they separate, are each equivalent in electrophoretic mobility to 18S rRNA. In some organisms the interaction between the halves is rather weak, so the total RNA preparation exhibits a single 18S-like rRNA band even on non-denaturing gel. In others the 28S rRNA is more robust, so it is still visible as a second band, but it rarely has twice the intensity of the lower one.

7- Compare between DNA and protein electrophoresis methods

Proteins are usually not run on agarose gels since the pores of the gel are too big to have a good separation. A 1% agarose gel has pores of around 150nm, poly acrylamide (which is usually used) have 3-6nm depending on the degree of polymerisation and gel strength. You will only use agarose gels for really high molecular weight proteins otherwise you won't see much separation of the sample. DNA samples have such a high molecular weight and can thus be separated on agarose gels

There are two important properties that determine how a molecule will move during gel electrophoresis:

- size and shape of the molecule
- charge of the molecule

You're applying an electric field, and all molecules will move according to their charge. The gel itself hinders the movement of larger molecules. This means that small molecules will move further than large molecules.

Nucleic acids like RNA and DNA have a uniform negative charge from the phosphate backbone. This means that they will generally separate according to their size and shape (plasmids can be present in different forms like supercoiled, circular or linearized).

Proteins have different charges, some have a positive charge at the pH typically used for gel electrophoresis, some have a negative charge, and some have no charge at that pH. So if you would simply run proteins on a gel, some would move backwards, some forward and some not at all.

To make the proteins separate according to their size we use the detergent SDS to denature the proteins, and to give them a uniform charge (SDS is negatively charged). Proteins are also run on polyacrylamide gels and not agarose as the size of the pores in agarose is a bad fit for the typical sized of proteins.

While I've never seen anyone using agarose for proteins, it seems that it is possible and sometimes used for larger proteins. I found one paper describing a protocol, they used very high concentrations of agarose in the range of 4-5%. I suspect that they won't handle well, but you should try that out yourself. If the proteins you're examining are a bit larger, using agarose should be possible.

8- Mention of the suitable gene transfer methods for plant and animal

The answer found in lecture 6

The answer of the second question:

Define these abbreviations and talk on each protocol in details

RFLP – AFLP – FISH – RAPD – QTL - PCR

RFLP:

Restriction fragment length polymorphism

In molecular biology, restriction fragment length polymorphism, or RFLP, is a technique that exploits variations in homologous DNA sequences.

the basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequenceoccurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which arecomplementary to the probe. An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis.

RFLP analysis may be subdivided into single- (SLP) and multi-locus probe (MLP) paradigms. Usually, the SLP method is preferred over MLP because it is more sensitive, easier to interpret and capable of analyzing mixed-DNA samples.[*citation needed*] Moreover, data can be generated even when the DNA is degraded (e.g. when it is found in bone remains.)

There are two common mechanisms by which the size of a particular restriction fragment can vary. In the first schematic, a small segment of the genome is being detected by a DNA probe (thicker line). In allele "A", the genome is cleaved by a restriction enzyme at three nearby sites (triangles), but only the rightmost fragment will be detected by the probe. In allele "a", restriction site 2 has been lost by a mutation, so the probe now detects the larger fused fragment running from sites 1 to 3. The second diagram shows how this fragment size variation would look on a Southern blot, and how each allele (two per individual) might be inherited in members of a family.

In the third schematic, the probe and restriction enzyme are chosen to detect a region of the genome that includes a variable VNTR segment (boxes). In allele "c"

there are five repeats in the VNTR, and the probe detects a longer fragment between the two restriction sites. In allele "d" there are only two repeats in the VNTR, so the probe detects a shorter fragment between the same two restriction sites. Other genetic processes, such as insertions, deletions,translocations, and inversions, can also lead to RFLPs. RFLP uses a much bigger sample of DNA than STR.

Analysis of RFLP variation in genomes was a vital tool in genome mapping and genetic disease analysis. If researchers were trying to initially determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease (see Genetic linkage). Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease, or who was likely to be a carrier of the mutant genes.

RFLP analysis was also the basis for early methods of Genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations.

Digestion, run and gel blotting

- Digest 3-5ug genomic DNA with 10Units of enzyme per ug of DNA. (Try to do reactions in a final volume of 15ul)
- Incubate samples at the suitable temperature for minimum 5 hours.
- At least 30’ before use, prepare a 0.6-0.8% agarose gel in 1x TBE, without EtBr. Use wide but thin combs. Don’t load the 2 most external lanes.
- Add blue juice to each sample (3ul) and load as much as you can(18ul).
- You can use 5ul of HindIII digested Lambda DNA (250ng) as size marker.
- Run the gel overnight at 70Volts (about 16 hours) in 1x TBE running buffer. No cooling is needed.

- Stain the gel in EtBr for 20', wash with tap water and take a picture putting a ruler beside the gel in order to estimate the distance run by the size marker.
- Put the gel in 0.25M HCl for 20'.
- Meanwhile set up the vacuum blot apparatus. Wash the porous support and wet the sealing rubber. Connect the vacuum pump to the vacuum trap and then to the apparatus.
- Wash the gel with millipore water.
- Put the nylon membrane onto the porous support, then the plastic mask (the mask must be slightly smaller then the gel), being very careful slide the gel onto the mask.
- Close the apparatus and start the pump (you should be able to see if vacuum is formed, if not look for the problem). Close the screws tighty and pour 1 litre of 0.4N NaOH. Apply a vacuum pressure of -50mm/H2O.
- Leave for 1 hour, checking from time to time that there is no leakage. If there is a small leakage add more NaOH to maintain the gel always submersed.
- Suck out the remaining NaOH and mark with a pencil the slots onto the nylon membrane.
- Remove the gel, and wash the membrane with 2X SSC to clean from agarose. Air dry the filter. Note with a pencil the date of the blot on the filter in an area covered by the mask. Cut to reduce the size of the membrane.

Labelling of the probe

- Boil for 5', 25 to 50ng of the DNA fragment to be labelled.
- Place on ice for 1'
- Set up the following labelling reaction. (**Pharmacia oligolabelling kit**)
    - 25 to 50ng denatured DNA (max 36ml).
    - 10ul provided reagent mix.
    - 3ul [alpha-32P]dCTP
    - 1ul Klenow enzyme

- Distilled water to 50ul
- Incubate at 37°C for 60’ at least.
- If the probe is not needed immediately it could be stored in the freezer for few days.
- Before use boil the probe for 5’. (Normally it is not necessary to get rid of the nucleotides.)

Pre-hybridisation and hybridisation

- If the membrane to be used is employed for the first time then an overnight pre-hybridisation is needed, otherwise 4-5 hours should be enough.
- Wet the membrane with either distilled water or 2x SSC. Drain off the excess of water or SSC and roll the membrane into the hybridisation tube. Add 20ml of hyb solution and 600ug of sonicated and denatured (boil for 5’) salmon sperm DNA (usually stock is 10ug/ul)
- Incubate in the rotating hybridisation oven at 65°C.
- After pre-hybridization, start hybridisation by adding the boiled labelled probe (NOT DIRECTLY on the membrane) to the pre-hybridisation.
- Incubate overnight.

Hyb solution:

1% SDS
1M NaCl
10% Dextrane Sulphate
make 1 litre with 50mM Tris-Cl pH 7.5
resuspend in waterbath at 65°C (You can store the solution in the freezer)

Washing of the membranes

- Add just a little(50-100ml) of 500ml of 2X SSC to the hyb solution.
- Mix for a couple of minutes then pour the solution into the radioactive waste tank.

- Put the hybridised membrane in a large box and pour the rest of the 500ml of 2XSSC.
- Shake at room temperature for 10 min (Be careful radioactivity is still high).
- Pour off the solution and replace with 500ml of pre-heated (65°C) 1X SSC + 0.1% SDS. Incubate for 20' at 65°C.
- Substitute the solution with pre-heated (65°C) 0.2X SSC + 0.1% SDS. Incubate while shaking for 20' at 65°C.
- Now you can measure the radioactivity level of your filter using the hand counter. The filter should not count more than 5 cpm. If it counts too much, wash at 65°C with 0.1X SSC +0.1% SDS for 20'.
- Wrap the membrane with Saran Wrap and expose.Leave at -80°C for 1 to 3 days.

Removal of the probe

- Put the membrane into boiling water +0.1% SDS, let slowly cool down at room temperature.
- Put the membrane into a plastic bag or wrap it with Saran Wrap and store in the fridge. (DON'T LET THE MEMBRANE DRY OUT).

AFLP Protocol

1. Restriction digest

For each reaction combine:

300 ng DNA

0.15 µl EcoRI NEB (20,000U/ml) = 3U

0.30µl MseI NEB (10,000U/ml) = 3U

4 µl 10x NEB#2 Buffer

0.4 µl BSA (10mg/ml)

ddH$_2$O to 40 µl with

incubate at 37oC for 1hr

PCR program: 'Dig-Lig'

1hr @ 37oC

3hrs @ 16oC

4oC

NEB = New England Biolabs

2. Ligation of adapters

2.1 Preparation of adapters

EcoRI DS adapter (60µl rxn, enough for 20 reactions)

1.7µl EcoRI adapter1 (1µg/µl) = 1.7µg

1.5µl EcoRI adapter2 (1µg/µl) = 1.5µg

3.0 µl 10x NEB#2 Buffer

53.8µl ddH$_2$O.

(This gives a final concentration of 5 uM)

EcoRI adapter1 5'-CTCGTAGACTGCGTACC

EcoRI adapter2 CATCTGACGCATGGTTAA-5'

MseI DS adapter (60µl rxn, enough for 20 reactions)

16µl MseI adapter1 (1µg/µl) = 16µg

14µl MseI adapter2 (1µg/µl) = 14µg

3.0 µl 10x NEB#2 Buffer

27.0µl ddH_2O.

(This gives a final concentration of 50 uM)

MseI adapter1 5'-GACGATGAGTCCTGAG

MseI adapter2 TACTCAGGACTCAT-5'

PCR program: '96X5MIN'

96°C for 5 min

Let the samples sit in the machine until temp drops to RT

Adapter sequences:

EcoRI adapter1 5'-CTC GTA GAC TGC GTA CC-3'

EcoRI adapter2 5'-AAT TGG TAC GCA GTC TAC-3'

MseI adapter1 5'-GAC GAT GAG TCC TGA G-3'

MseI adapter2 5'-TAC TCA GGA CTC AT-3'

2.2 Ligation

After 1hr incubation (Dig-Lig), add to each 40 µl restriction digest:

3.0 µl EcoRI DS adapter

3.0 µl MseI DS adapter

1.0 µl 10X Ligase Buffer

0.5 µl T4 ligase NEB

2.5 µl ddH_2O

Total volume per reaction is now 50 µl. Continue reaction for 3 additional hours at 16°C in the thermocycler (program 'Dig-Lig').

3. PreAmplification

PCR Buffer:

500ul Tricine Taq buffer (300mM Tricine, 500mM KCl, 20mM MgCl2, pH to 8.4 w/ KOH)

500ul 50% (w/v) Acetamide

200ul H2O

dNTP mix:

1ul each 100mM dNTP, 46ul H2O

For 20ul rxn:

10.8ul H2O

4.8ul PCR Buffer

1ul dNTP mix

0.4ul EcoRI+1 primer (10uM)

0.4ul MseI+1 primer (10uM)

0.6ul Taq polymerase

2ul digestion-ligation product

PROGRAM: AFLP1

1: 72oC 2min

2: 94oC 1min

3: 94oC 0:30s

4: 56oC 0:30s

5: 72oC 1:00s

6: repeat 3-5 35X

7: 72oC 2min

8: 4oC

Run agarose gel to make sure you see a smear with size ~1kb and smaller

Dilute PreAmp 1:20 with sterile H_2O

Primer sequences:

EcoRI+1 primer 5'-GAC TGC GTA CCA ATT CA-3'

MseI+1 primer 5'-GAT GAG TCC TGA GTA AA-3'

4. Selective Amplification

4.1radio-labeling of EcoRI primer (for radioisotope detection)
for 12.5ul rxn:

5 μL gamma-32P-ATP (~3,000 Ci/mmol) or 2.5ul gamma-33P-ATP (~2,000Ci/mmol)

1.25 μL 10xT4-kinase buffer (NEB)

0.5 μL T4-kinase (NEB)(10 units/μL)

H2O to 10ul

2.5 μl of EcoRI primer (50ng/ul: 4.42ul stock primer solution(100uM)+45.58ul H2O)

PCR program: 'LABEL'

1: 60 min @ 37°C,
2: 10 min @ 70oC
3: 4oC

This gives a labelled primer with a concentration of 10 ng/μL.

4.2 Selective amplifications

for 20ul rxn:

H2O to 20ul

4.8ul PCR Buffer (see pre-amplification)

1ul dNTP mix (see pre-amplification)

0.4ul fluorescent-labeled EcoRI+3 primer (10uM) or 2.4ul radio-labeled EcoRI+3 primer (10ng/ul)

0.4ul MseI+3 primer (10uM)

0.6ul Taq9

2ul 1:20 dilution of pre-amplification

PROGRAM: AFLP2

1: 94oC 1min

2: 94oC 0:30s

3: 56oC 0:30s

4: 72oC 1:00s

5: repeat 3-5 35X

6: 72oC 2min

7: 4oC

5. 3730 sequencer run

Mix:

890ul H2O (8.9ul per well)

10ul Liz ladder (0.1ul Liz ladder per well)

Distribute 9ul of the mix and add 1ul 1:10 dilution sample

FISH Protocols

A. Fixation

The fixation of the sample is one of the most critical steps in the protocol. A good fixative

should preserve the cell morphology while concomitantly permeabilizing all cells for the

labeled oligonucleotide. Standard fixatives are aldehydes and alcohols.

Formaldehyde is reacting slowly, therefore the fixation can be finetuned according to the

specific needs. For many microorganisms, good results are achieved by fixation at final

concentrations between 1% - 4% formaldehyde over night at 4°C. Alternatively 1-2 h at room

temperature may also suffice for good fixation. After the fixation a dehydration series of 50,

80, and 96% ethanol may help to permeabilize cells for FISH.
The quality of the formaldehyde solution is critical. Ideally it should be freshly prepared

from paraformaldehyde. We often store fresh prepared PFA under a nitrogen atmosphere.

• 4% PFA preparation:

1. pour 2 g of paraformaldehyde (PFA) powder in 50 ml phosphate buffered

saline (PBS; 130 mM NaCl, 10 mM Na2HPO4/NaH2PO4, pH 7.4) or a similar

buffer (use mask for weighing PFA – irritant if inhaled)

2. heat to app. 60° C (must not boil!), until suspension is clear (app. 1/2 h); if not,

add some drops of 1N NaOH

3. check pH and adjust to pH 7.0

4. filter through 0.2 µm filter and place on ice

Usable for up to 1 week, and up to 6 month if stored dark under nitrogen atmosphere

at RT.
Formalin, a concentrated 35-37% formaldehyde solution, can be readily used as well, if the

solution is fresh (means, no precipitates at the bottom of the bottle). Below you find protocols

for fixing pure cultures with (1) Gram-negative, (2) Gram-positive cell wall, for (3) planktonic

cells and for (4) sediment/soil samples.

1. Fixation of pure cultures with Gram-negative cell wall (Amann et al., 1990)

1. harvest cells during logarithmic growth by centrifugation of an aliquot (ca. 2 ml) in

microcentrifuge, (10 min. at 4000 x g)

2. discharge supernatant and resuspend cells in 750 µl PBS (145 mM NaCl, 1.4 mM

NaH2PO4, 8 mM Na2HPO4, pH 7.4)

3. fix by adding 250µl of a 4% PFA fixative (1% final concentration)

4. incubate for 1 (for thick walled cells) to 24 h (for fragile cells) at 4°C

5. pellet cells by centrifugation (10 min. at 4000 x g), discharge supernatant

6. thoroughly resuspend fixed cells in 500 µl PBS

7. repeat step 5 and 6

8. add 500 µl absolute ethanol and resuspend cells thoroughly

9. at this stage samples can be stored at -20°C for several months

2. Fixation of pure cultures with Gram-positive cell wall (Roller et al., 1994)

1. harvest cells during logarithmic growth by centrifugation of an aliquot (ca. 2 ml) in

microcentrifuge, (10 min. at 4000 x g)

2. discharge supernatant and wash cells in PBS

3. pellet cells by centrifugation (10 min. at 4000 x g), discharge supernatant

4. add 500 µl PBS, resuspend cells thoroughly

5. add 500µl cold, absolute ethanole, mix

6. at this stage samples can be stored at -20°C for several months

No PFA used (pure ethanole fixation) because the thick cell wall of the Gram-positive

bacteria can become completely impenetrable, otherwise.

3. Fixation of planktonic samples (Glöckner et al., 1999)

1. Add formalin (37% formaldehyde) to a water sample to a final concentration of 1-3%

and fix for 1-24 h at 4°C; needs to be optimized for new sample types.

2. Place a moistened support filter (0.45 µm pore size, cellulose nitrate, 47 mm diameter;

Sartorius, Germany) and a membrane filter (0.2 µm pore size, white polycarbonate, 47

mm diameter; Millipore, Eschborn, Germany; shiny side up!) into a filtration tower;

filter a known volume of the fixed sample by applying gentle vacuum; support filters

may be utilized for several samples; for cell numbers of around 106

ml-1, 10 ml of

sample is generally sufficient.

3. After complete sample filtration, wash with 10-20 ml of sterile H2O; remove H2O by filtration, put the membrane filter in a plastic petri dish, cover and allow air-drying.

4. Store at -20°C until processing; filters can be stored frozen for several months without apparent loss of hybridization signal.

4. Fixation of sediment / soil samples (Llobet-Brossa et al., 1998)

1. Fix sediment samples with fresh formaldehyde (end concentration 1 - 4%) for 1 -2 at RT or max 24 hours at 4°C;

2. centrifuge at 16.000 g for 5 minutes; pour off supernatant and resuspend sample with

1 X PBS pH 7.6;

3. Repeat step 2 twice;

4. Store sediment sample in a 1:1 mix of PBS / ethanol at -20°C or -80°C until further processing.

Random Amplified Polymorphic DNA (RAPD)

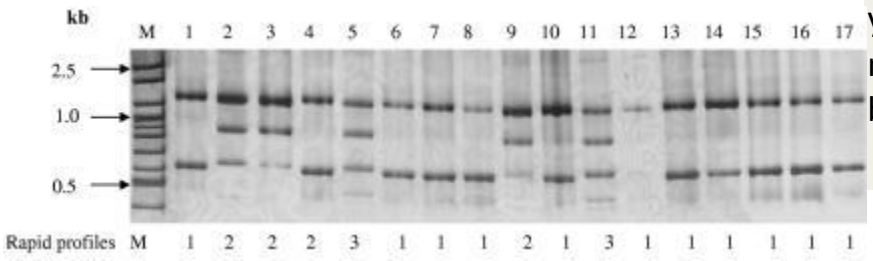
Introduction

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.

How It Works

Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

Example



RAPD is an inexpensive yet powerful typing method for many bacterial species.

Silver-stained polyacrylamide gel showing three distinct RAPD profiles generated by primer OPE15 for *Haemophilus ducreyi* isolates from Tanzania, Senegal, Thailand, Europe, and North America.

Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains.

Limitations of RAPD

- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
- PCR is an enzymatic reaction, therefore the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible.
- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

Developing Locus-specific, Co-Dominant Markers from RAPDs

- The polymorphic RAPD marker band is isolated from the gel.
- It is amplified in the PCR reaction.
- The PCR product is cloned and sequenced.
- New longer and specific primers are designed for the DNA sequence, which is called the **Sequenced Characterized Amplified Region Marker (SCAR).**

Standard PCR protocol

Introduction

Taq DNA Polymerase is a thermostable enzyme derived from the thermophilic bacterium *Thermus aquaticus*. The enzyme is in a recombinant form, expressed in *E. coli*. It is able to withstand repeated heating to 95 °C without significant loss of activity. The enzyme is approximately 94 kDa by SDS-PAGE with no detectable endonuclease or exonuclease activity. It has 5'→3' DNA polymerase activity and 5'→3' exonuclease activity. Each lot of *Taq* DNA Polymerase is tested for PCR amplification and double-stranded sequencing. The enzyme is supplied at 5 units/µL and comes with an optimized 10x reaction buffer.

Unit Definition: One unit incorporates 10 nmol of total deoxyribonucleoside triphosphates into acid precipitable DNA in 30 minutes at 74 °C.

Reagents

Select Taq DNA polymerase based upon user's preference:

Standard Taq DNA Polymerase

Containing MgCl2		Separate MgCl2	Readymix- premixed PCR mastermix with buffer and dNTPs	
Clear formulation without dye	With red dye for direct load on gels	Clear formulation without dye	With red dye for direct load on gels	Clear formulation without dye
Taq DNA Polymerase from Thermus aquaticus (D1806)	REDTaq® DNA Polymerase (D4309)	Taq DNA Polymerase from Thermus aquaticus, without MgCl2 (D4545)	REDTaq® ReadyMix™ PCR Reaction Mix (R2523)	ReadyMix™ Taq PCR Reaction Mix (P4600)

- PCR grade water (**W1754**)
- Primers diluted to working concentration (10µM working stocks are sufficient for most assays)
- Oligos → **Order Custom Oligos here**
- DNA to be amplified
- Dedicated pipettes
- Thermal cycler
- Sterile filter pipette tips
- Sterile 1.5 mL screw-top microcentrifuge tubes (such as **CLS430909**)
- PCR tubes, select one of the following to match desired format:
  - Individual thin-walled 200 µL PCR tubes (**Z374873** or **P3114**)
  - Strip tubes, 200uL (**Z374962**)
  - Plates
    - 96 well plates (**Z374903**)
    - 384 well plates (**Z374911**)
  - Plate seals
    - AlumaSeal® 96 film (**Z721549**)
- dNTP mix, 10 mM each of dATP, dCTP, dGTP, and dTTP (**D7295**, not needed for readymixes)

Procedure

The optimal conditions for the concentration of *Taq* DNA polymerase, template DNA, primers, and $MgCl_2$ will depend on the system being utilized. It may be necessary to determine the optimal conditions for each individual component. This is especially true for the *Taq* DNA polymerase, cycling parameters, and the $MgCl_2$ concentration. It is recommended the enzyme and the $MgCl_2$ be titrated to determine the optimal efficiency.

1. Select appropriate table for reaction setup: standard or readymix reagent. Add the reagents to a appropriate sized tube in the order provided in the table. For large number of reactions, a mastermix without the template should be setup

and aliquoted into reaction tubes. At the end, template should be added to appropriate tubes.

Standard PCR reaction

Amount	Component	Final Concentration
w µL	Water	
5 µL	10x PCR Buffer (P2192, B5925 or P2317)*	1x
1 µL*	Deoxynucleotide Mix	200 µM
w µL	Forward primer (typically 15-30 bases in length)	0.1-0.5 µM
x µL	Reverse primer (typically 15-30 bases in length)	0.1-0.5 µM
0.5 µL	*Taq* DNA Polymerase (D6677 or D5684)*	0.05 units/µL
y µL	Template DNA (typically 10 ng)	200 pg/µL
z µL	25 mM MgCl2 (use only with buffer P2317)	0.1-0.5 mM
50 µL	**Total reaction volume**	

*Note: Components listed are reagents sold as D1806, D4309 or D4545

Readymix PCR reaction

Amount	Component	Final Concentration

25 µL	Readymix (R2523 or P4600)	
w µL	Forward primer (typically 15-30 bases in length)	0.1-0.5 µM
x µL	Reverse primer (typically 15-30 bases in length)	0.1-0.5 µM
y µL	Template DNA (typically 10 ng)	200 pg/µL
z µL	Water	
50 µL	**Total reaction volume**	

2. Mix gently by vortex and briefly centrifuge to collect all components to the bottom of the tube.

3. Add 50 µL of mineral oil to the top of each tube to prevent evaporation if using a thermal cycler without a heated lid.

4. The amplification parameters will vary depending on the primers and the thermal cycler used. It may be necessary to optimize the system for individual primers, template, and thermal cycler.

Typical cycling parameters:

25-30 cycles of amplification are recommended

Denature template	94 °C	1min

Anneal primers	55 °C	2 min
Extension	72 °C	3 min

5. The amplified DNA can be evaluated by agarose gel electrophoresis and subsequent ethidium bromide staining. Mineral oil overlay may be removed by a single chloroform extraction (1:1), recovering the aqueous phase.

- See more at: http://www.sigmaaldrich.com/technical-documents/protocols/biology/standard-pcr.html#sthash.tKZdFiQJ.dpuf

The answer of the third question:

1-Compare between old and modern sequencing methods (short notes)

Sanger's method and other enzymic methods

1 Random approach 171

2 Direct approach 171

3 Enzyme technology 175

4 Sample preparation 175

5 Labels and DNA labelling 176

5.1 Radioisotopes 176

5.2 Chemiluminescent detection 176

5.3 Fluorescent dyes 177

6 Fragment separation and analysis 180

6.1 Electrophoresis 180

6.2 Mass spectrometry – an alternative

4. Maxam & Gilbert and other chemical methods

5. Pyrosequencing – DNA sequencing in real time by the detection of released PPi

6. Single molecule sequencing with exonuclease

The four best known techniques of DNA sequencing are reviewed and close to 200 references cited. These techniques are the Sanger method, the Maxam & Gilbert method, the Pyrosequencing™ method, and the method of single-molecule sequencing with exonuclease. There are good prospects for the emergence of new and non-conventional methods of DNA sequencing, which may one day revolutionize the field of DNA sequencing. Some of these candidates are methods based on atomic force microscopy, on the use of nanopores or ion channels, on quantum optics, DNA microarrays and TOF MS of aligned ssDNA fibres, among others (some of them are reviewed by Marziali & Akeson, 2001). All these new possibilities deserve a special review paper with a deep and critical analysis.

2- Mention of the methods of creating competent cells (short notes)

The Inoue Method for Preparation of Competent E.Coli

“Ultra-competent” Cells

Buffers & Solutions

1. High quality DMSO

2. .5M PIPES (piperazine-1,2-bis[2-ethanesulfonic acid]) at pH 6.7

i. Dissolve 15.1g of PIPES in 80mL Milli-Q H2O

ii. Adjust pH of solution to 6.7 (with KOH or HCl)

iii. Add Milli-Q H2O to final volume of 100mL

iv.

(optional: sterilize solution by vacuum filtration in pre-rinsed Nalgene filter of

.45µm pore size)

3. Inoue Transformation Buffer (chill to 0˚C before use)

i. Dissolve solutes below in 800mL of Milli-Q H2O

Reagent Qty/L Final Conc.

MnCl2·4H2O 10.88g 55mM

CaCl2·2H2O 2.20g 15mM

KCl 18.65g 250mM

ii. Add 20mL PIPES (.5M, pH 6.7)

iii. Adjust total volume to 1L with Milli-Q H2O

iv.

Sterilize Inoue transformation buffer by filtration through pre-rinsed .45µm

Nalgene filter

Media

1. LB or SOB for initial culture growth

Centrifuge & Rotors

1. Sorvall GSA rotor or equivalent

Special Equipment

1. Liquid nitrogen

2. Polypropylene tubes

3. Shaking incubator (18˚C)

4. Water bath at 42˚C

5. Chilled microfuge tubes

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Mc

Clean Lab Protocol

Day 1: Growing Bacterial Cultures

1.

Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated

for 16-20 hours at 37˚C.

2.
Transfer colony into 25mL of LB broth or SOB medium in at 250mL flask.

3.
Incubate culture for 6-8 hours at 37˚C with vigorous shaking (250-300rpm).

4.
Inoculate three 1L flasks of 250mL SOB using the below volumes of this st arter culture.

Flask # Vol. of Starter Culture

1 1mL

2 50µL

3 25µL

5.
Incubate all three flasks overnight at room temperature (18-22˚C) with mo derate shaking.

Day 2: Harvesting Cells and Freezing Competent Cells

1.
Read the OD600 of all three cultures. Continue to monitor every 45min un til reading is at 0.55.

2. Transfer the culture vessel to ice water bath for 10min.

3.

Harvest cells by centrifugation at 2500g (3900 rpm in Sorvall GSA rotor) fo r 10 minutes at RT in 50mL falcon tube.

4.

Pour off medium and dry the tube (inverted) on paper towels for 2min (u se vacuum aspirator to remove any drops of remaining medium adhering t o walls of the centrifuge bottle or trapped in its neck).

5.

Resuspend the cells gently (by swirling) in 80mL of ice-cold (0˚C) Inoue tra nsformation buffer.

6.

Harvest cells by centrifugation at 2500g for 10 minutes at room temperat ure.

7.

Pour off the medium and dry the tube on paper towels for 2min (use vac uum aspirator

to remove any drops of remaining medium adhering to walls of the centri fuge bottle or trapped in its neck).

8. Re-suspend cells GENTLY in 20mL of Inoue transformation buffer (0˚C).

9. Add 1.5mL of DMSO (swirl to mix bacterial suspension).

10. Store on ice for 10min.

11.
Quickly dispense 50µL aliquots of suspensions into chilled,sterile microfuge tubes.

(20mL of suspension -> 400 tubes of 50µL).

12.
Snap freeze competent cells in liquid nitrogen (store stock at -80˚C). Janua ry 2010

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Clean Lab Protocol

13.
When needed,remove tube of competent cells from freezer, thaw in hand, use immediately.

3- Write about making cDNA libraries (short notes)

Preparation of Electrocompetent E. coli (i.e. DH5α)

Step 1. Preferably, select single colony of E. coli from fresh LB plate for inoculating a 10 ml 2XYT overnight (O/N) starter

culture. Alternatively, streak out frozen glycerol stock of bacterial cells onto LB plate, grow plate O/N, and then select

single colony for starter culture. Grow 10 ml starter culture O/N in 37°C shaker (250rpm).

Step 2. Inoculate 1L of 2XYT media and place culture in 37° shaker. Grow cells and measure OD600 every 45min-1hr. When

the OD600 equals 0.6-0.9 (log phase growth), remove the cells from the shaker and place on ice.

NOTE: It very important to keep the cells at 4°C (or on ice) for the remainder of the procedure.

Step 3. Split the 1L culture into four equal parts by pouring ~250ml of culture into each chilled 250ml Corning pointed bottle.

Step 4. Spin (#1) in GPR centrifuge at 4000rpm, 25min at 4°C.

(if you chose to use the J6/ JS-4.2 rotor (E. Davidson Lab), use 1L bottles , fill half full, spin 4000rpm, 20min, at 4°C.)

Step 5. Place bottles on ice. Remove supernate immediately as cell pellet begins to lift off quickly. Gently resuspend each

pellet in 200ml ice-cold dH20.

Step 6. Spin (#2) in GPR centrifuge at 4000rpm, 25min at 4°C.

Step 7. Place bottles on ice. Remove supernate. Gently resuspend each pellet in 100ml of ice-cold dH20.

Step 8. Spin (#3) in GPR centrifuge at 4000rpm, 25min at 4°C.

Step 9. Place bottles on ice. Remove supernate. Gently resuspend each pellet in 20ml ice-cold 10% glycerol. For each pair of

250ml Corning bottles, transfer both 20ml cell suspension into one chilled 50ml conical tube- therefore you should end

up with two 50ml conical tubes on ice where each tube contains ~40ml of cells in 10% glycerol.

Step 10. Spin (#4) in GPR centrifuge at 4000rpm, 10min at 4°C.

Step 11. Place tubes on ice. Remove supernate. Gently resuspend each cell pellet in 1ml of ice-cold 10% glycerol. Final

OD600 of resuspended cells » 200-250.

Step 12. With cell suspensions on ice, prepared 70l aliquots of cells in pre-chilled 1.5ml eppendorf tubes. Snap freeze tubes

containing cells in liquid N2. Store frozen cells at -80°C.

NOTE: liquid N2 very hazardous- use caution and don't contact N2 directly!

Electroporation of Electrocompetent E. coli (i.e. DH5)

Step 1. Locate Electroporator power source and cuvette holder. (Bio-Rad). Set the conditions for transformation according to strain.

For DH5a cells, use 25 mFD, 200 W, and 1.8 kV. The time constant (tau value) should be 3-4 msec.)

For either TG2 or JE2 strains of E. coli, use 25 mFD, 200 W, and 2.5 kV (time constant = 4.6-4.8 msec).

Step 2. Thaw required number of frozen cell aliquots (each tube 70l = two transformations) on ice.

Thaw plasmid DNA in TE/H20 on ice.

Place 15ml conical tube containing 10ml of 2XYT media without antibiotics on ice.

Step 3. Place 3λ of DNA along wall of 0.2cm cuvette. Pipet 35 λ of thawed electrocompetent cells onto DNA drop. Flick cuvette to settle DNA + cells mixture into bottom of cuvette.

Step 4. Have 1ml pipette containing 1ml of 2XYT media ready. Dry off any moisture from cuvette outside and immediately place cuvette in white plastic holder. Slide holder into position and zap cells. If you hear a high constant tone, immediately add the 1ml of 2XYT to cells! Transfer cells from cuvette into 1.5ml eppendorf tube and store on ice until step 5.

The tone indicates that you have successfully electroporated your cells. Record the time constant value. Repeat procedure for remaining samples.

If you see or hear sparking coming from your cuvette of cells, then the cells are dead! Repeat that sample again.

Things that can cause sparking: excess water on cuvette outside, human skin oil on cuvette outside, too high salt conc. in DNA sample (try diluting DNA 10-fold), and poorly made electrocompetent cells.

Step 5. Outgrow transformed cells in eppendorf tubes by incubating the tubes in 37°C water bath for 1-1.5hrs.

Step 6. Hi/Lo plate transformed cells onto LB + Amp plates. Expect ~108 transformants per 1µg of DNA.

Hi plate: Plate 200l of outgrown transformed cell suspension.

Lo plate: Spin remaining 800l of cells in microfuge for 20-30sec to pellet cells. Gently shake open tube over sink. The volume of liquid that clings to tube is roughly 150-200l. Resuspend cells in this volume of retained media and plate cells (using all the volume) on to LB + Amp plates.

Step 7. Place LB + Amp transformant plates in 37°C bacterial incubator for 16-24 hrs until colonies appear.

The answer of the fourth question:

Write about the role of these enzymes in biotechnology

Reverse transcriptase: build cDNA (first strand) based on mRNA template

Taq DNA polymerase: build DNA based on DNA template

DNA ligase: joint between two nucleotides found in the same strand of DNA or makes phosphodiester bond between two nucleotides.

Hind III: type of endonuclease enzymes

BamHI: type of endonuclease enzymes

Proteinase K: degrades the protein peptides

Dnase: digests DNA strands

Rnase: digests RNA strands

Lysozyme: degrades the cell wall

We wish good luck to all

Dr. Mahmoud Mokhtar