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REVIEW ARTICLE

MOLECULAR HYBRIDIZATION TECHNIQUES OF NUCLEIC ACIDS

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Abstract: The nucleic acid hybridization is the process wherein two DNA or RNA single chains (mono-stranded) from different biological sources, make the double catenary configuration, based on contingent sequence homology of the two sources, resulting DNA-DNA, RNA-RNA or DNA-RNA hybrids. The purpose is identification or localization of certain nucleic acid sequences (genes) in the genome of some species. The target molecule representing the DNA, RNA or protein sequence that should be identified or located and the probe molecule who identify the target, by hybridization. Hybridization on a solid carrier is: Southern blotting, Northern blotting, Western blotting. Hybridization stages is: probe synthesis, probe marking (radioactively or non-radioactively), target DNA processing, target DNA denaturation, target DNA transfer to solid carrier, molecular hybridization.

Keywords: blotting, target, probe, primers, renaturation, vectors.

Introduction

Biosphere should be considered a commodity borrowed from the next generations, that should be returned in best conditions. Regardless the attitude of some groups of political and economic interests, that control the uninformed people, it is clear it will be impossible to feed the next generations (according to demographic forecasts, the next generations will be increasingly frequent) only with resources ensured by conventional technologies. Under the global circumstances, the genetic engineering techniques and especially the recombined DNA technology, gains more and more ground within modern technologies (Vassu *et al*, 2002). Identification, isolation and sequentialisation more easily and accurately of genes that codify various characters showing economic interest, is currently carried out by molecular hybridization techniques as well as *in vitro* and *in vivo* molecular cloning techniques (Chen *et al.*, 1998, Yeung *et al.*, 2002).

Results achieved by applying the hybridization methods are expressed in homology percentage. A reassociation percent higher than 70-80% proves conspecificity. It has been set that organisms within species should have at least 70% DNA homology by hybridization and at most 5% sequence divergence. This is the definition of "genomic" species in case of prokaryota (Stackebrandt and Goebel, 1994, Rossello-Mora and Amann, 2001). The studies of

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DNA hybridization have changed several times the conventional bacterial systematics, but it is compulsory to be joined by another technique, in order to be confirmed. For measurements, test strains from the surveyed taxon are recommended to be used, otherwise interpretation errors might occur (Vassu *et al*., 2002, Stoica *et al*., 2002).

mtDNA (mitochondrial DNA) homology studies are carried out by comparing restrictions patterns and has the advantage that, DNAmt having smaller dimensions than the nuclear DNA, the analysis of restriction sequences is easier. Querol, 1996 developed a technique whereby the total cell DNA is treated with restriction endonucleases that recognize tetranucleotidic (H*ae*III, H*pa*, R*sa* I, M*bo*I etc.) or pentanucleotidic (H*inf*I, M*ae*III, D*de*I, etc) sequences for which there are lots of sites in the nuclear DNA (that is thus broken in many small dimension sequences) and a few in mtDNA (that will divide the few large dimension sequences, that are easily emphasized in the agarose gel). The RFLP (*Restriction Fragments Length Polymorfism*) profiles thus obtained enable emphasizing of various strains, having a special taxonomic value (Su and Meyer, 1991, Duffner and O'Connell, 1994).

Of all existing molecules in live systems, rRNA (ribosomal RNA) is the best molecular timer, as it shows a very high degree of operational constancy in the evolutionary scale. This "molecular timer" behaviour has driven the elaboration of another model of phylogenetic development of life on Earth (Woese, 1990 states that organisms belong to 3 different fields: *Bacteria, Archaea* and *Eukarya*). The 16S rRNA from prokaryota (with 1500 ribonucleotides that form 3 different fields and 50 helixes) as well as 5S rRNA or 23S rRNA, is the best parameter of estimating the phylogenetic affinity degree between two bacterial strains (Kurtzman, 1992, Kolbert *et al*., 1999, Vaughan *et al*., 1999). Ribotyping is characterization of strains using stably maintained traits such as the essential rRNA genes, located on the chromosome, allows the reliable and reproductible typing of isolates (Duffner and O'Connell, 1994). (For eukaryota the rDNA genes are reiterative units (100-200 copies/genome) arranged in tandem and is a perfect material for the restriction and taxonomy reactions based on RFLP. At present, GenBank, EMBL-Bank, that are powerful databases, have a complete nucleotide sequence for over 20000 RNAr molecules. The rRNA-rDNA hybridization is extremely laborious and can be carried out only if nucleotidic sequence similarity is around 80% (Wuyts *et al*., 2002).

The ARDRA (***A**mplified **R**ibosomal **D**NA **R**estriction **A**nalysis*) (Vanneechoutte *et al*., 1995) complete the phylogenetic studies. Moyer introduces the OTU (***O**perational **T**axonomic **U**nits*) concept representing a group of micro-organisms with identical ARDRA pattern in case of using at least 3 restriction endonucleases. Depending upon the combination of restrictases used, the ARDRA technique is capable to make a difference among bacterial genra, among congeneric species and even among conspecific species and, together with RFLP are the most accurate taxonomic detection systems (Moyer *et al*., 1996).

The ASO method (***a**llele **s**pecific **o**ligonucleotide assay*) ensures detection of pointlike changes of DNA sequences by differential hybridization of the sequence analysed with oligonucleotide sequences (probes) complementary with conventional and mutant sequences. The key condition is the accurate konwledge of the two sequences (conventional and mutant). The stages of the method are: development typical to analysed sequence (PCR - ***p**olymerase **c**hain **r**eaction*); sequence transfer on a membrane and its denaturation; hybridization with conventional sequence complementary probe; hybridization with mutant sequence complementary probe.

In situ hybridization is quite modern and enables localization and local analysis of DNA or RNA, with applications in the cell RNA analysis and concerning major chromosomal changes. The sensitivity of the technique is such that threshold levels of detection are in the region of 10-20 copies of mRNA per cell. Monitoring microbial diversity and function at the single-cell level is critical for understanding the ecological role of microorganisms in aquatic environments, for example (DeLong *et al*. 1989, Ramsing *et al*., 1996). **F**luorescent ***in situ*** **h**ybridization (FISH) methods with phylogenetic probes have been widely used to identify specific prokaryotic cells in various natural communities. FISH is a straightforward and established method, which is mainly used for taxonomic or phylogenetic

identification of bacterial cells within microbial communities (Bagasra *et al*., 1995, Vaughan *et al*., 1999). *In situ* molecular detection of bacterial cells in mixed culture, will become more powerful when coupled with other advanced technologies such as digital image processing and flow cytometry (Schwarchzacher and Heslop-Harrison 2000, Chen and Hodson, 2001).

Hybridization principle

The nucleic acid hybridization is the process wherein two DNA or RNA single chains from different biological sources, make the double catenary configuration, based on nucleotide complementarity and of contingent sequence homology of the two sources, resulting DNA-DNA, RNA-RNA or DNA-RNA hybrids. In most cases, the purpose of the hybridization techniques is identification or localization of certain nucleic acid sequences in the genome of some species. Two basic notions are used: the target molecule representing the DNA, RNA or protein sequence that should be identified and the probe molecule who identify the target, by hybridization. When hybridization takes place on a solid carrier is named blotting and is divided in 3 categories:

- Southern blotting whereby DNA molecules are identified using DNA or RNA probes;
- Northern blotting whereby RNA molecules are identified using RNA or DNA probes;
- Western blotting whereby proteic sequences are identified using specific antibodies.

The general characteristics of nucleic acid probes:

- To show a chemical marking to allow viewing the target probe molecular hybrid;
- To have a dimension of 10-10000 nucleotides. Too short probes hybridize very quickly (some minutes), but they show non-specific hybrids, too and are difficult to trace. Too long probes hybridize very slowly (some hours), but hybrids are more stable and more specific;
- Not to show intracatenary complementarity areas, not to hybridize with itself or with non-targets, but to have a high sequence homology specificity with the target molecule.

Hybridization stages

1. Probe synthesis

1.1. Probe synthesis by "nick-translation" (figure 1) usually starts from a target molecule copy thus:

- the DNA molecule d.s. (double stranded) is subject to **DN-ase I** action that is a non-specific site endonuclease and that takes to pieces randomly, phosphodiesteric bundles, in the presence of Mg^{2+} ions, generating single chain breaks = "nicks";
- as a result of exonuclease 5'-3' action of a **DNA polymerase I** from *E. coli* nicks are enlarged;
- quasisimultaneously with exonuclease 5'-3' action of DNA polymerase I, this shows its 5'-3' polymerization activity using marked dNTPs and thus a translation of the single chain break is carried out ("nick-translation");
- DNA d.s. sequences thus obtained and marked uniformly are subject to thermal denaturation by breaking the hydrogen bridges and obtaining single chain probes.

1.2. Probe synthesis using randomized primers. Randomized primers are heterogenous sequence oligonucleotides that can hybridize in many sites of the matrix chain. They can be compared or can be obtained thus :

a) DNA is isolated from the salmon sperma or calfe timus and is acted over it with **DN-ase I** so as a high population of single chain DNA oligonucleotide sequences are obtained having dimensions of 6-12 nucleotides.

b) By means of an automatic oligonucleotide synthesizer having all the 4 types of dNTPs in the reaction medium.

The stages of probe synthesis method using randomized primers are (figure 2):

- Thermic denaturation of the matrix and production of DNA single chains,

- Attaching of randomized primers in various sites of single chains (at random).
- Using 3 types of simple nucleotides and one marked radioactively, in the persence of **DNA polymerase I** and **Klenow-DNA pol I** (it has no exonuclease 5'-3' activity), completion of holes among primers, by copying the matrix information,
- By heat denaturation radioactively marked probes are obtained (Monedero *et al.*, 1997).

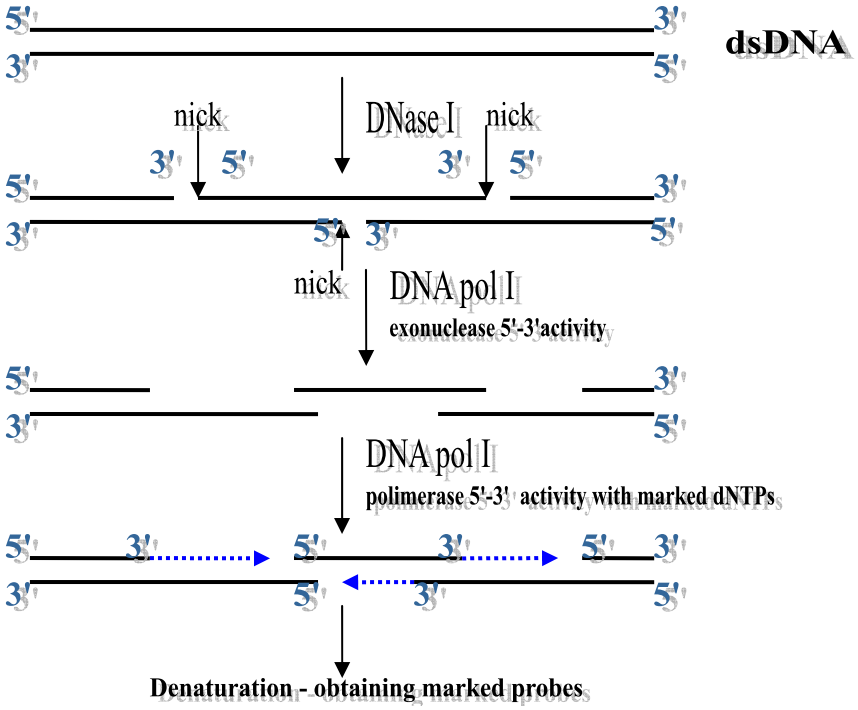

***Figure 1**. Probe synthesis by "nick-translation"*

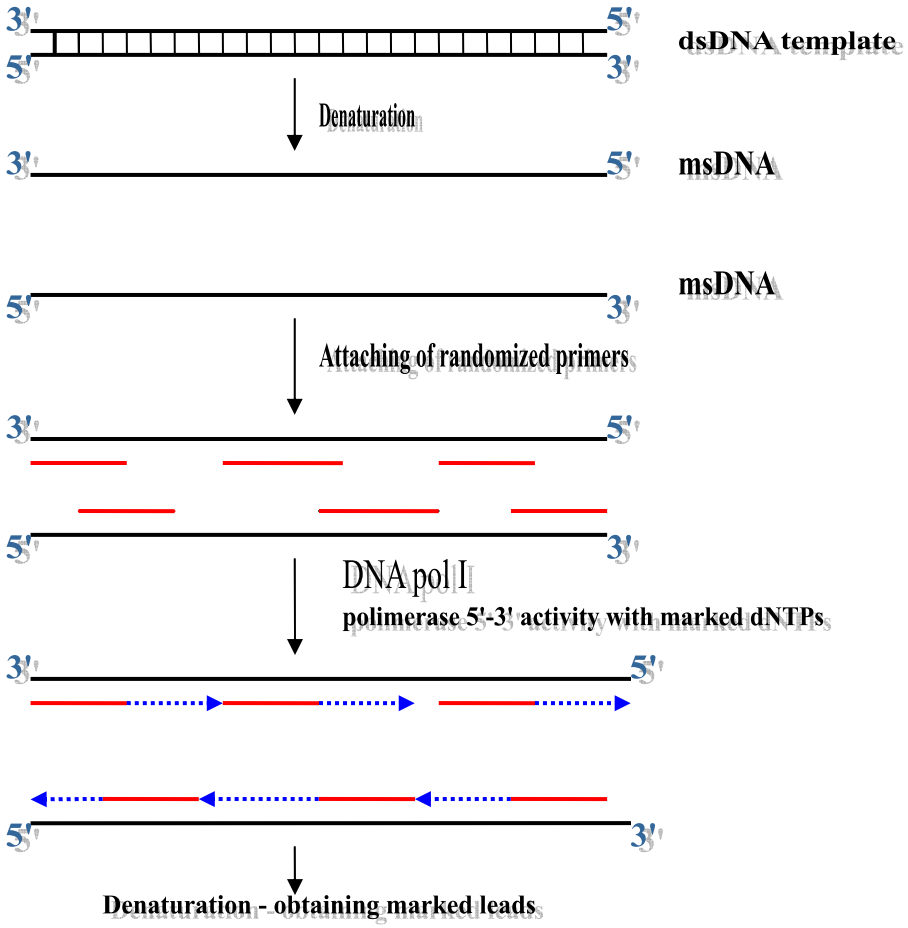

Figure 2. *Probe synthesis using randomized primers*

1.3. Total DNAc probe synthesis (complementary DNA with an m.s.RNA population) can be done in two versions:

- Total mRNA is isolated from cells and secondary structures are taken to pieces by heating at 70°C, for 5 min. Randomized primers (upstream and downstream) are attached and polymerization is started from them, in the face of dNTPs, Mn^{2+} ions and a reverse-transcriptase (rTth or RT isolated from the murine leukemia virus – MLV or from the avian mieloblastosis virus – AMV) (figure 3). A RNA-DNA hybrid thus results that is denaturated by NaOH treatment at a temperature of over 70°C and thus short "partial" DNAc probes are obtained (Chaillou *et al*., 1998b, Chaillou *et al*., 1998c).

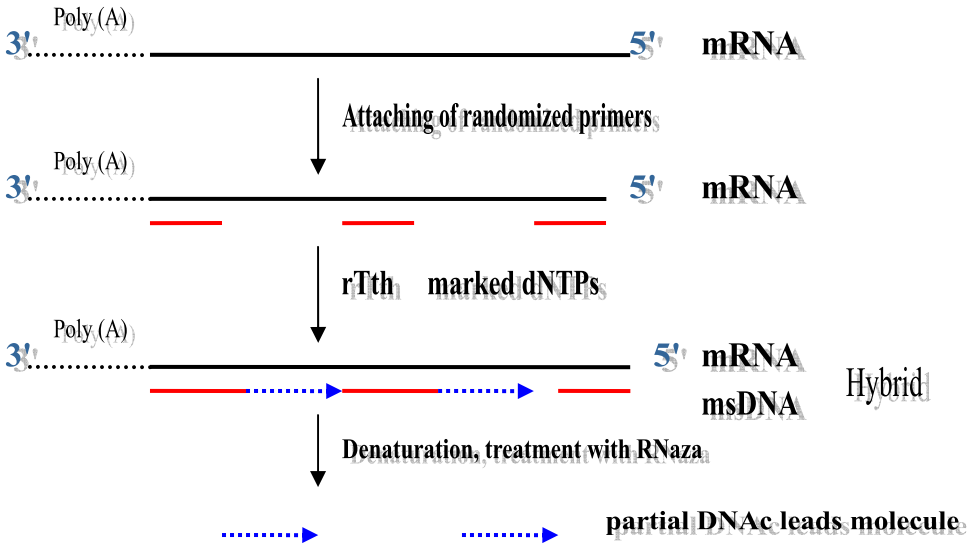

Figure 3. *DNAc probe synthesis with randomized primers*

- As regards a total mRNA isolated from cells and denaturated, oligo(dT) primers are attached based on complementarity to poly ends (A) from 3'ends of mammalian mRNA molecules. The remaining stages are identical with those of the former version, but finally integral DNAc probes are obtained (for those that fully copied the mRNA molecules by revers-transcriptase) (figure 4).

1.4. RNA probe synthesis by *in vitro* registration. It requests exogenous DNA cloning (based on it obtaining of RNA probes is requested) in clonation vectors that contain strong promoters, highly specific such as SP-6, T3 or T7 phage promoters. These promoters are recognised extremely specific by RNA polymerase from phages concerned (and that it does not recognize neither chromosomal bacterial or plasmidial promoters nor eukariota promoters recognized by other RNA polymerase). Vectors containing these promoters are plasmidial type (pGEM 3/4 or pGEM 3z/4z) or phagemides: pBluescript (figure 5) or vectors derived by the λphage (λZAP) (Chaillou *et al.*, 1998a, Chaillou *et al.*, 1998b). The stages of the technique are:

- Recombined vector containing in MCS (**m**ulty**c**loning **s**ite) the exogenous DNA is linearized with a restriction endonuclease that cuts outside the two high specificity promoters. For instance, if a pBluescript phagemide is used for restriction, B*st* H II endonuclease produces blunt ends. Thus d.s.DNA sequences are obtained containing T3 and T7 promoters at ends, and inside the exogenous DNA is obtained and based on it, formation of RNA probes is targeted.

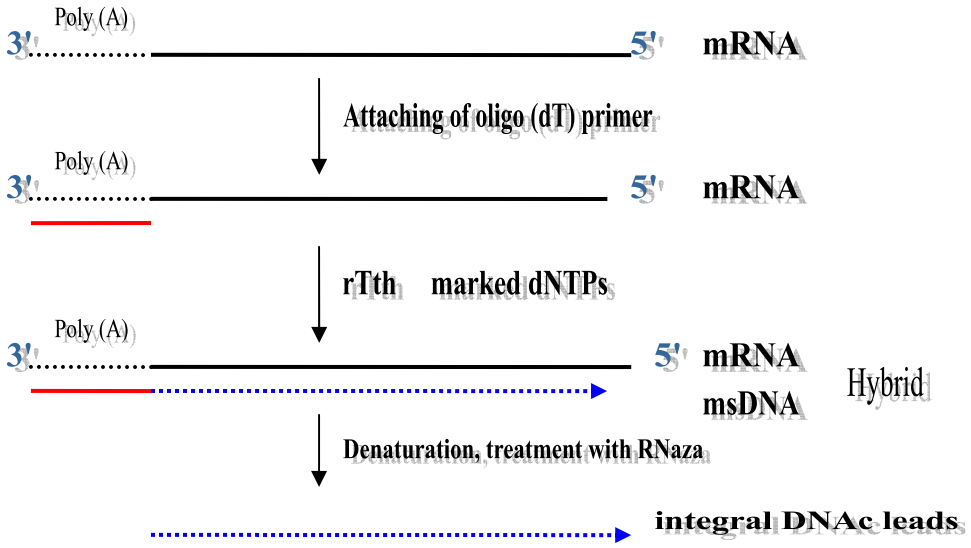

Figure 4. *DNAc probe synthesis with oligo(dT) primers*

- Sequences are mixed with T3 and/or T7 RNA polymerase with all types of rNTPs of which one is marked radioactively) and with or spermidine or bovine serum albumin (that intensify *in vitro* registration by 2-2.5 times). Reaction mixture is maintained 1-2 hrs at 37°C and marked RNA probes are obtained.
- DNA molecules, that were used as matrix in registration, are degraded by DN-ase I, and RNA probes are precipitated with ethanol 100% in the presence of ammonium acetate 2.5M.

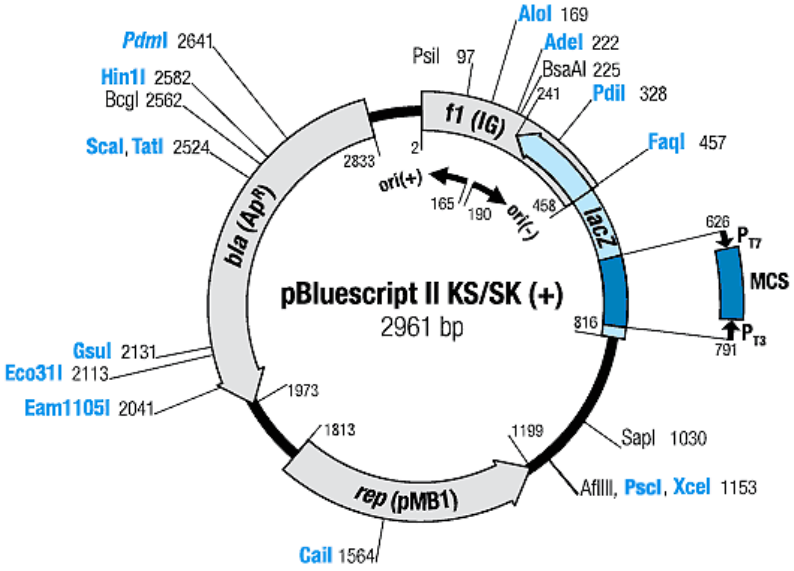

Figure 5. *Genetic map of pBluescript phagemide*

2. Probe marking is carried out several times **radioactively** during the probe synthesis using ^{32}P or ^{35}S. The method has the advantage that very small amounts of nucleic acids can be detected. The following disadvantages are mentioned: risk of handling radioactive materials, imbalance of isotopes and long exposure times. Apart the incorporation of radioactively marked nucleotides in new-synthesized chains, are marked the 3'OH smooth or cohesive ends of d.s.DNA sequences, resulted after restrictases activity.

- The 5'PO_4^{2-} cohesive ends, made by most restrictases, are "filled" by attaching radioactively marked nucleotides, at 3'OH end (shorter), based on complementarity with the nucleotides "read" on the 5'PO_4^{2-} end (longer). Reaction takes place *in vitro* in the presence of the Klenow-DNA pol I enzyme from *E. coli* (figure 6).
- The blunt ends produced by some restrictases (H*pa* I, S*ma* I, H*ind* II, H*ae* III) are marked by adding some radioactive nucleotides at 3'OH end, in the presence of an enzyme named terminal-dNTP-transferase (shorter - terminal transferase) (figure 7). This is a special DNA polymerase, extracted from veal thymus, (more precisely from prelymphocytes or from very early stages of lymphoid distinction) and that in the presence of Mg^{2+} ion it adds a purine to the 3'OH end, and in the presence of Co^{2+} ion it adds a pyrimidinic nitrate base at the same end of the DNA chain (Walker *et al.*, 1996).

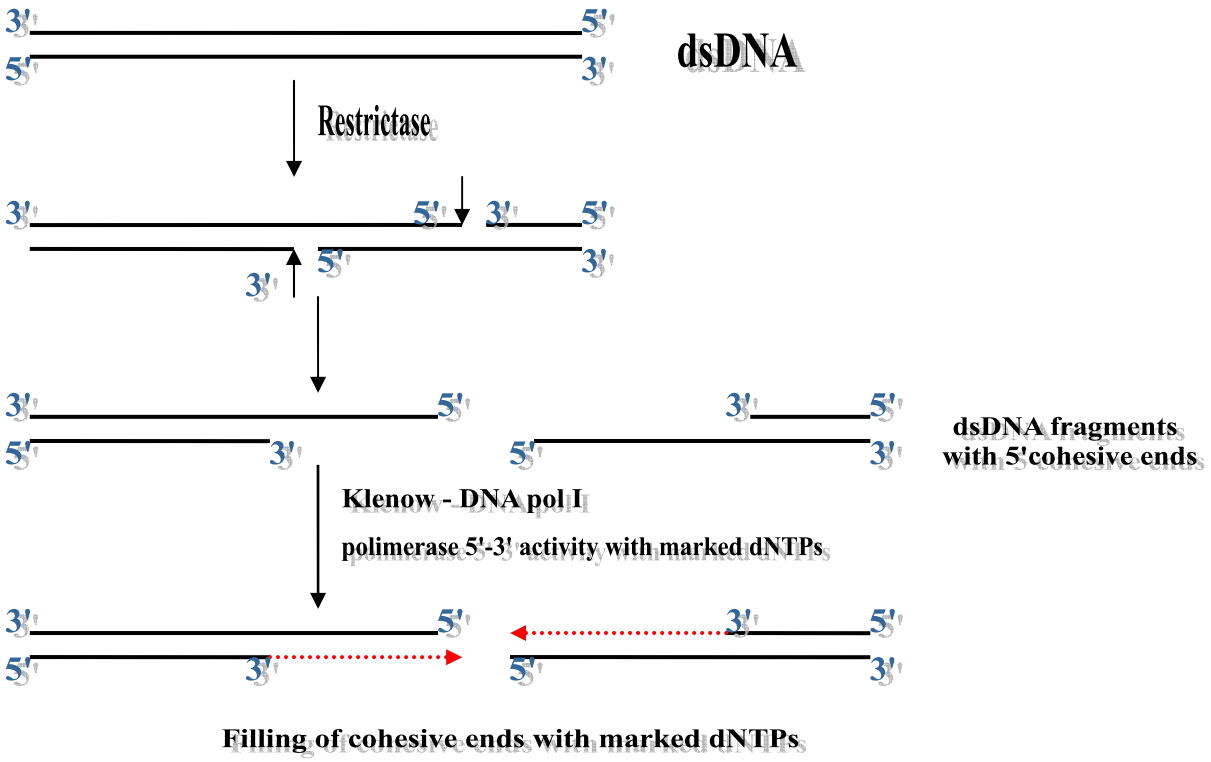

Figure 6. *Radioactively marking of probe with 5'PO_4^{2-} cohesive ends*

Non-radioactive marking uses reporter molecules that are directly or indirectly detectable. Such reporter molecules are: fluorochromes, biotine (H vitamine) coupled with avide or with streptavidine or digoxigenine (DIG-labeled DNA probe) coupled with antibodies-antidigoxigenine (Monedero *et al.*, 1997, Gösseringer *et al.*, 1997).

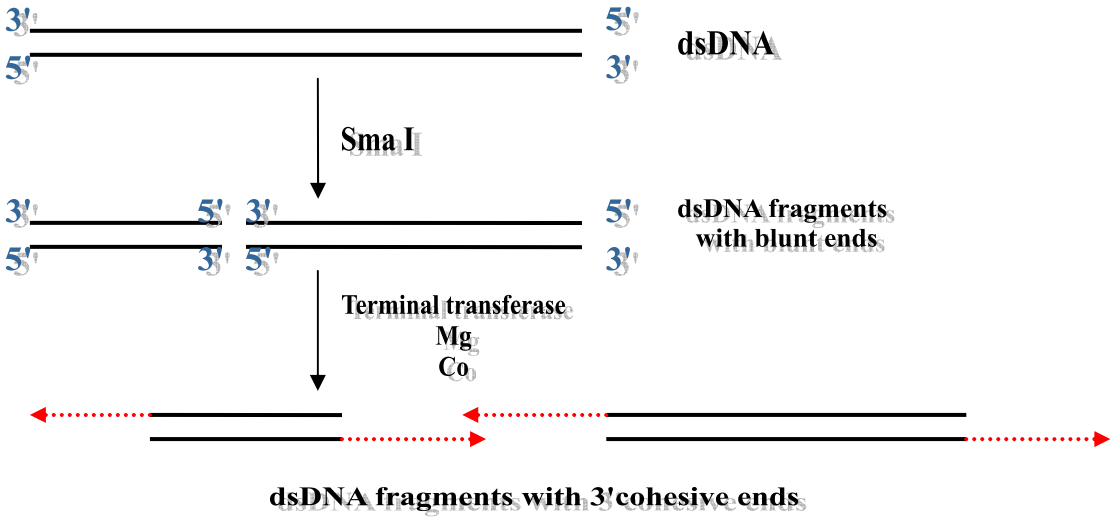

Figure 7. *Radioactively marking of probe with blunt ends*

In case of fluorescent marking of nucleic acid probes duplicates of nucleotides are used coupled with fluorescein (fluorescein-dUTP) that incorporates in chains by standard enzyme techniques and that can be directly or indirectly detected (when fluorescein is used as heptene) (Grundy *et al*, 1993, Saier, 1996).

Biotine is incorporated in nucleic acid probes as biotine-dUTP through a standard enzyme reaction. Afterwards hybrids obtained by molecular hybridization will be detected by coupling biotine with avidine from the avidine-alkali phosphatase (or avidine-peroxidase from horse radish). Avidine is a glycoproteine from egg-white and has 4 subunits and each of it is coupled by a biotine site. Enzyme (alkali-phosphatase or peroxidase) will degrade a chromogen compound and will give a color reaction (blue, red-brown respectively).

3. Target DNA processing assumes the source DNA isolation and purification and its digestion with a suitable restrictase so as the target oligonucleotidic sequence is obtained as a linear, double-stranded molecule. Sequences obtained by the restriction reaction are separated by electrophoresis in the agarose gel.

4. Target DNA denaturation in order to get single chain (mono-stranded) molecules (by breaking hydrogen bridges between complementary nucleotides) means the introduction of electrophoresis gel in NaOH 0.5N solution. The breaking of phosphodiesteric bundles between complementary nucleotides of the two chains of DNA can be made by HPLC (***H**igh **P**erformance **L**iquid **C**hromatography*), the accurate but laborious and expensive method that can provide errors by mixing the nuclear DNA with the mitochondrial one (in case of eukaryota). Another method is the chromosomal DNA ultracentrifugation in gradient of CsCl when the floating density of the sample is compared with the reference DNA sample and resulted value depends on %G +C :

Floating density =1.66 + 0.098 · % mole GC

The most common denaturation method is thermal denaturation (Stoica *et al*, 2002, Vassu *et al.*, 2002). Increasing the temperature of the reaction mixture between 20-100°C, absorbance variation at a wave length of 260nm (A_{260}) is checked by spectrophotometry 260nm (A_{260}). During denaturation absorbancy can raise by 40% as well. This process is called hyperchromic shift. Tm is the melting temperature where half of DNA is as a single chain type and half is still bicatenary. Calculation formulas vary according to ionic charge

of the buffer used. For prokaryota two correlation equations have been elaborated :

%mole GC=2.44 · Tm – 169 (when the buffer is 0.1x SSC with a high concentration of Na^+ ions)

%mole GC= 2.44 · Tm – 131.5 (when the buffer is TE, so a low concentration of Na^+ ions).
For yeasts the following equation is valid :

%mole GC= 2.44 · Tm –106.4 (Owen, 1985)

By means of a SWIFT – Tm v1.05 software, the values of Tm are calculated with the analysis of hyperchromic shift curves, using a reference DNA from *E. coli* where %GC= 51 is known. %GC is currently accepted as a quasiuniversal molecular taxonomy parameter and it had a major contribution to phylogenetic studies.

5. Target DNA transfer to solid carrier (nitrocellulose paper, nylone membrane) cand be carried out in three ways (Monedero *et al.*, 1997, Vassu *et al.*, 2002):

- Transfer of DNA sequences from the gel, through capillarity by an SSC buffer, in a sandwich system (kitchen paper laid on and under the solid carrier). The transfer rate depends on the size of sequences and agarose concentration.
- Electrophoretic transfer is carried out only on nylon membranes that are electrically charged and this method is also effective for small DNA molecules up to 60pb.
- Vacuum transfer is much more effective and faster than the other versions. It assumes the electrophoresis gel is laid directly on the solid carrier, and the entire assembly is laid on a spongy carrier under which there is a vacuumed chamber. The buffer is poured over the assembly and then it is extracted by vacuum and takes over the nucleic acid from the gel and stores them on the solid carrier.

The solid carrier, with nucleic acids stored on it, is exposed to UV radiations, and as a result, are created cross-links between nucleic acids and carrier structure, thus provide the fastening.

6. Molecular hybridization consist in immersion of the solid carrier with the target single chain DNA molecules in 5x SSC buffer containing the marked probe as well and are left in contact 14-16h. After hybridization probe remained non-hybridized is removed by serial washing with an SSC buffer. Renaturation time also depends on %moleGC thus :

T_{OR}= (0.51 · %moleGC) + 47 (where OR is optimum renaturation)

In the competitive reassociation apart the non-marked target m.s.DNA and having a long length and marked probe m.s.DNA and having a small length, a reference m.s.DNA having a long length is used. After hybridization and washing, filters are read with a Geiger-Muller meter. A high number of impulses means a low homology degree, and a low number of impulses – a high homology degree between the target DNA and the probe DNA. For example, Chaillou and contributors use the Southern hybridization performed with the *Lactobacillus pentosus xylR* gene (encoding the D-xylose repressor protein) as a probe revealed the existence of a *xylR* homologue in *Lb. brevis* (Chaillou *et al*, 1998a).

The process is influenced by 5 factors:

- Length of target DNA sequences (the bigger the higher renaturation rate is, but a long length drives a higher solution viscosity, and as a result, renaturation is suppressed; a compromise was made: the target DNA sequences should have a uniform molecular weight of $1\text{-}5\cdot10^5$ Da);
- Ionic strength of incubation buffer (Na^+ ions from reaction buffer affects denaturation and renaturation as it influences nuclease activity);
- DNA preparation purity (RNA, carbohydrates, solution proteins interferes with hybridization);
- Initial concentration of DNA molecules (C_0) and incubation time (t):

$C/C_0= 1/(1+ k \cdot C_0t)$;

- Optimum incubation temperature of the reaction mixture is 25°C. At lower temperatures non-specific hybrids are formed, by reassociations between non-homologue sequences, at high temperatures, the reaction rate decreases a lot.

Conclusions

1. Identification, isolation and sequentialisation more easily and accurately of genes that codify various characters with economic interest, is currently carried out by molecular hybridization techniques.
2. Together with RFLP and ARDRA techniques, molecular hybridization is the most accurate taxonomic detection system who is capable to make a difference among bacterial genra, among congeneric species and even among conspecific strains.
3. *In situ* hybridization and the ASO method ensures detection of pointlike changes of DNA sequences or major chromosomal changes in prokaryota or eukaryota genome. These techniques have important economical and medical implications in food industry, agriculture, pharmaceutics diagnosis and treatment of genetic diseases.

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