Lecture 14 - PCR Applications and Lab Practicum (AMG text pp. 159-169)
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Diagnostic Applications of PCR

There are three primary diagnostic applications of PCR:

- detecting pathogens using genome-specific primer pairs
- screening specific genes for unknown mutations
- genotyping using known STS markers

Multiplex PCR can be used to simultaneously screen for pathogenic and non-pathogenic strains of E. coli using strain-specific primer pairs. By using at least two strain-specific primer pairs for each organism, and replicate sample reactions, it is possible to reliably detect the presence of >10 genome equivalents. In this example, two food samples were analyzed for the presence of a specific pathogenic strain of E. coli. By using appropriate control samples for the two bacterial strains, it can be seen that food sample 1 is contaminated.

The use of quantitative Real-Time RT-PCR to detect viral genomes such as HIV (AIDS) or HPV (human papilloma virus in association with cervical cancer) is another diagnostic application of PCR.

Identifying genetic mutations
The PCR technique of single strand conformational polymorphism (SSCP) is one of the most widely used methods for detecting single base pair changes in genomic DNA. SSCP is based on the principle that two single strand DNA molecules of identical length, but unlike sequence, will migrate differentially in a non-denaturing acrylamide gel. This is due to sequence-dependent intramolecular folding reactions which contribute to the overall structure of the molecule.

In this example, AMG primer pairs were used to detect nucleotide alterations in a specific region of the AMG gene known to co-segregate as dominant mutation. Using DNA from three individuals (two disease heterozygotes and a homozygote normal), PCR amplification was performed with AMG-specific primers that flank a region where multiple dominant alleles have previously been identified.

A variation on this assay system is the WAVE DNA Fragment Analysis System by Transgenomic. In this method the DNA sample is heated and cooled, but by cooling slowly under optimal hybridization conditions, both homoduplex and heteroduplex PCR products will form. The sample is then run through a high performance liquid chromatography (HPLC) system, and OD260 peaks are resolved as they are eluted from the column under gradient conditions. This Denaturing HPLC (DHPLC) system is used here at the University of Arizona and is available as a core service through the LMSE.
PCR genotyping using sequence tagged sites

STSs (Sequence Tagged Sites) are defined as genomic DNA segments for which sequence information and physical mapping data are available. These markers can be defined by PCR primer pairs which can be used to generate distinct PCR products. Many of the STS markers that have been used for pedigree and forensic genotyping are VNTRs (Variable Nucleotide Tandem Repeats), and STRPs (Short Tandem Repeat Polymorphisms) and SNPs (Single Nucleotide Polymorphisms). PCR primers that specifically amplify across polymorphic repeat sequences can be used to determine genotype based on the type of PCR products generated.

Laboratory applications of PCR

PCR is a valuable laboratory tool that is used routinely for tasks that can be done other ways, but which are easier and quicker when using PCR. While only a few laboratory applications of PCR are described here, there are literally hundreds of variations of these published methods. Two general applications have been chosen as representative examples; 1) subcloning DNA targets using PCR, and 2) PCR-mediated in vitro mutagenesis.

Subcloning DNA targets using PCR

There are numerous situations in which a specific DNA segment needs to be cloned into a plasmid vector, but there are no convenient restriction enzymes sites to facilitate the design of a subcloning strategy. Three strategies are commonly used to subclone PCR products:

• T/A Cloning
• Restriction Site Addition
• Blunt-end Ligation

The most common method is restriction site addition which utilizes PCR primer pairs that incorporate restriction enzyme recognition sites into the 5’ end of the oligonucleotide. Another method is to take advantage of the propensity of Taq polymerase to spuriously add a dATP residue to the 3’ terminus of extended chains. A specially-prepared dT cloning vector is often used that includes lacZ coding sequences for colony screening. Blunt-end cloning of PCR products can be done using a lacZ plasmid vector that has been digested with an appropriate enzyme such as SmaI.

PCR-mediated in vitro mutagenesis

Mutagenic PCR is a random mutagenesis technique that exploits the elevated error rate of Taq polymerase in the presence of MnCl2 and high MgCl2. Mutagenic PCR is similar to random chemical mutagenesis with the added advantage that the chosen primer pair provides a convenient way to target the random bp mutations to a defined segment of DNA. Strategies have also been developed that permit the construction of unique gene fusions using consecutive PCR reactions that utilized the PCR products of one reaction to prime DNA synthesis during the second amplification. This technique is sometimes called PCR-mediated gene SOEing (splicing by overlap extension).
Amplification of differentially-expressed gene sequences
Differential screening and subtraction hybridization are two techniques that can be used to isolate gene transcripts which are present at different levels in two RNA populations. However, these approaches require large amounts of RNA to synthesize sufficient quantities of an enriched cDNA probe for library screening. In contrast, RT-PCR can be used to generate cDNAs from very small amounts of mRNA. This section describes the three basic RT-PCR cloning approaches that have been used to identify differentially-expressed genes.

- Differential display reverse transcriptase PCR (DDRT-PCR)
- Suppression subtraction hybridization (SSH)
- Amplification of cell-specific transcripts using RT-PCR

Differential display reverse transcriptase PCR (DDRT-PCR)
The key to DDRT-PCR is the use of modified oligo dT primers for reverse transcription that anneal to a subset of poly A+ mRNAs due to differences in dinucleotides at the 3’ end of the primer. DDRT-PCR generates subsets of cDNA products based on differences in 3’ dinucleotides and the location of 5’ arbitrary priming sites for PCR. Schematic diagram showing how a 5’-dT-GC-3’ primer is used to synthesize cDNA from a subset of RNAs containing the appropriate 3’ dinucleotide adjacent to the poly A tail. Of these first strand cDNAs, only a fraction will serve as appropriate templates for separate PCR reactions containing a known arbitrary primer.

A representative autoradiogram is shown on the left from a DDRT-PCR gel that contains reactions using RNA from cells that were untreated (-) or treated (+) with a steroid hormone. The representative Northern blot on the left demonstrates how a candidate DDRT-PCR product needs to be verified as a differentially-expressed sequence using conventional RNA assays.

Suppression subtraction hybridization (SSH)
The basis for differential amplification in SSH is two-fold. First, conditions are used that promote rapid reassociation kinetics with excess “driver” cDNA sequences to normalize two “tester” cDNA pools. Following this driver-tester hybridization reaction, the two tester pools are mixed and the unhybridized single strand low abundance molecules are allowed to anneal under conditions that favor duplex formation. In the subsequent PCR amplification step, these unique “tester” duplexes are exponentially amplified by a pair of adaptor primers. Second, the PCR amplification of non-differential cDNAs is suppressed because these undesirable sequences contain inappropriate priming sites, or are capable of forming intrasstrand “panhandle” structures that are poor substrates for PCR.

Amplification of cell-specific transcripts using RT-PCR
Lab Practicum 6 in the textbook is a hypothetical strategy based on previously published work describing the cloning of olfactory receptors using the method of single cell RT-PCR. Within the last two years, a molecular neurobiology lab led by Charles Zuker at UC San Diego actually used this approach to clone putative mammalian taste receptors. The molecular genetic strategy was basically the same in both cases and led
to the identification of distinct families of highly related G Protein Coupled Receptors (GPCRs).

**Note:** This is a good example of how to design your own Lab Practicum Class Presentations - use combinations of known molecular genetic methods in to accomplish the research objective.

**Hey Bud, How Does it Taste?**

**Research Objective**
A large food processing conglomerate has recently acquired a small biotechnology company that specializes in molecular genetic neurobiology. The gustatory research team at the parent food company found that humans can taste many more flavors than previously known. The gustation researchers used high resolution human taste tests to map three new flavor-associated neuronal impulses to cell subsets in the tongue. The research team has decided to use RT-PCR and differential screening to identify genes expressed exclusively in the three distinct lingual regions.

**Available Information and Reagents**

1. Needle biopsies have been collected from the three mapped flavor zones (TX, TY, and TZ) using high resolution taste tests that were performed on five volunteers. Cell samples (~5,000 cells/sample) have been prepared from all 15 biopsies (TX1-5, TY1-5 and TZ1-5).

2. An EcoRI-ApaI-oligo dT primer, called EAT24, with the nucleotide sequence 5'-ATTGGAATTCCTGAGGGCCC(T)24 -3', has been purified for use in the RT-PCR reaction and 10 mg of EcoRI-cleaved lambda ZapII vector has been treated with calf intestinal alkaline phosphatase.

3. A cDNA probe corresponding to the 3' end of Tng1 (a tongue-specific developmental marker) has been prepared to use as hybridization control in Southern blots that will be used to verify putative flavor zone-specific transcripts identified by differential cDNA library screening.

**Basic Strategy**
The researchers hypothesize that flavor-specific receptors will be expressed at moderately high levels (~1% of the mRNA) in cells from the three lingual regions and that the 3' sequence of the TX, TY and TZ receptors will be different. The EAT24 primer will be used to initiate first strand cDNA synthesis in cell extracts prepared with a reverse transcription lysis buffer containing RNase inhibitors. The 3' end of single strand cDNA products will be poly (A)-tailed with terminal deoxynucleotidyl transferase (TdT), and 30 cycles of PCR will then be performed with the EAT24 primer under appropriate buffer conditions.
The RT-PCR cDNA products will be split into three portions:

1) cDNA that will be cleaved with EcoRI and cloned into the ZapII vector to create 15 separate cDNA libraries.

2) cDNA that will be combined into separate TX, TY or TZ cDNA pools and used for differential library screening.

3) cDNA that will be saved for use as volunteer-specific cDNA probes on Southern Blots.

Triplicate filter lifts will be made from each master plate and screened with cDNA probes representing pooled TX, TZ and TY RT-PCR products. Plaques that hybridize most strongly with the homologous, but not heterologous, T-mixed probes will be isolated (e.g., plaques from a TX library that hybridize with the TX mixed probe but not the mixed TY or TZ probes).

Candidate inserts identified in the differential cDNA screening step are then analyzed by Southern blot filters that are sequentially hybridized to RT-PCR probes from individual volunteers. The Tng1 cDNA sequence is used as an internal control on these Southern blots to confirm that the individual RT-PCR cDNA samples contain sufficient levels of this ubiquitous tongue transcript.

As shown in this example, the TZ1.Zb and TZ1.Ze inserts hybridize to transcript probes present in all three volunteer TZ samples, suggesting that these correspond to cell-specific gene products.

Comments
There are three major assumptions that need to be met for this strategy to work.

1. The TX, TY and TZ cell types have to contain gene transcripts that are totally unique for the differential screening to be informative.

2. These putative cell-specific transcripts have to be expressed at a sufficiently high levels to permit screening of only a small portion of the library and to constitute a moderate amount of the probe.

3. The most significant difference in gene expression profile needs to be directly related to the distinct cell phenotype.

Prospective
The most meaningful cDNA sequence information would be derived from the gene transcript ORF, and therefore, additional cDNA cloning would be necessary since the RT-PCR products in this scheme are biased to the 3’ terminus (this strategy uses oligo dT primer to selectively amplify poly A+ mRNA). Two methods could be used to obtain 5’ coding sequence. One would be 5’ RACE and the other would be to simply screen a standard cDNA library.
It would also be important to identify the putative TX, TY and TZ cell types in the human tongue using in situ hybridization with the cell-specific cDNAs. This would need to be done with pathology samples, and depending on the sensitivity of the assay and type of sample, in situ RT-PCR may be required. The longer term objective of a project like this would likely require the isolation of mouse TX, TY and TZ genes for the development of genetic models to dissect the relative contribution of each receptor subtype to flavor "perception".

Study Questions

1. What is the purpose of making 5 individual libraries from the 5 TX patient samples, why not just pool the TX cDNA and screen it?

The strategy behind this approach requires that each cell-specific transcript be uniquely expressed - and that its very presence in a sample is evidence of its relevance. Since this type of cell-specific sensory receptor is usually expressed at high levels (>1% of the mRNA), it does not require a full library screening. Therefore, by finding the most abundant cell-specific TX transcripts (i.e., clones that are not detected by the TZ and TY probes) in each of the five libraries, it makes it more likely that these TX genes represent biologically relevant findings (all humans would be expected to express the same sensory receptors).

2. What is the reason for screening the same plate using triplicate filters and three probes, only one of which is the homologous probe (TX library screened with not only the TX probe, but also the TY and TZ probes)?

This screening strategy is used to eliminate clones that are expressed in all three cell types. For example, a plaque that hybridizes equally well with all three probes is most likely a housekeeping gene or a tongue gene that does not correspond to cell-specific taste receptors. In contrast, plaques that hybridize uniquely to the homologous probe will have a greater chance of being what you are looking for.

3. How are the Southern Blots used to further characterize candidate clones? What cDNA material is run on the gel, where does probe come from?

Since the library screening utilizes one volunteer sample for each library, but the probe used to screen the library is a mixed probe from pooled samples, it is important to show that the candidate cDNA insert is actually present in all five individuals - this can be done by hybridizing the cDNA inserts (material run on the gel), with probes made from the individual samples (material used for each sequential screening of the filter; volunteer samples 1-5).

4. What would you do once you identified candidate cDNAs that met all of your criteria based on hybridization patterns?

Obtain the DNA sequence and run a homology search against GENBANK, you would expect to see some type of membrane receptor motif if it has the type of function you predict. In this case, one would predict GPCR homologies.