Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Nucleic Acid Amplification: Alternatives to PCR

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Slide 1:
Hello, my name is Tim Uphoff. I am the section head for Molecular Pathology at Marshfield Labs in Marshfield, WI. Welcome to this Pearl of Laboratory Medicine on "Nucleic Acid Amplification: Alternatives to PCR." I initially considered a title for this talk to be: "How many ways are there to skin a cat?"

Because this is such a broad subject, we could spend many hours covering these methods. This discussion will not be all-inclusive of all possible permutations but my objective is to provide a brief overview of some of the most common alternatives to PCR. You can always google any of these methods and find much more in-depth information.

Slide 2:
If you would like to learn more about PCR, Dr. Katie Bennett has put together an excellent presentation on Real-Time PCR, which I highly recommend (her Pearl is available at www.traineecouncil.org).

All of the methods I will discuss today have some similarity with PCR because they all rely on the ability of complementary nucleic acids to hybridize and form a double-stranded molecule. For most of these methods, the double-stranded molecule then serves as the starting point for some type of polymerase to initiate replication.

I have divided the methods into two categories: Target specific and target non-specific methods. The target specific methods I will discuss have been primarily employed for infectious disease applications. The target non-specific methods result in amplification of all the starting material and are used primarily in Whole Genome Amplification [WGA].

While I will mention some of the more obscure methods, I will focus on those which have been utilized in commercial clinical tests.

Slide 3:
Both BCA and Hybrid Capture (HC) methods provide sensitive quantification without amplification of the target nucleic acid.

Branched DNA was used in early viral load assays produced by Bayer and is depicted on the next slide.

Hybrid Capture is used in the Digene [now Qiagen] Hybrid Capture 2 HPV assay. HC is analogous to ELISA but in this method DNA-RNA hybrids are recognized by specific antibodies and detected with alkaline phosphatase utilized for chemiluminescent detection.

Slide 4:
In this figure, we see three different target genes labeled 1,2, and N hybridized to capture probes attached to individual beads. This and each hybridization step below is followed by a wash step to remove unhybridized probe.

- Dual specificity label extenders hybridize to the target and the Pre-Amplifier (PreAmp) molecules.
- Then, multiple Amplifier (Amp) molecules hybridize to each PreAmp.
- Finally, multiple Label Probe oligonucleotides hybridize to each Amp.

The addition of streptavidin phycoerythrin (SAPE) generates a signal that is proportional with the amount of target DNA present in each sample.

Slide 5:
Now let's move on to probe amplification.

As we discuss Probe and Target amplification moving forward, keep in mind that each method must utilize some method of repeatedly generating single-stranded target so that a probe or primer may anneal to begin a new round of replication. Some use heat denaturation and thermocycling while others are isothermal and achieve this enzymatically.

Ligase chain reaction [LCR] sounds comparable to PCR and is very similar but with a few critical differences.

This method was used in early Abbott lab CT/NG assays.

- The target is shown above in a single-stranded form as a thick blue line.
- The probes are shown as adjacent thin lines annealed to the top of the target and unbound below.
- The gap between the two probes [indicated by an arrow above] is filled by DNA polymerase and DNA ligase joins the two probes together.
- Through temperature cycling steps similar to PCR, we can complete further denaturation, hybridization, and elongation/ligation steps.
- The ligated probes can serve as template for subsequent rounds of amplification.
- Thus, we can obtain exponential amplification of the ligated probes which can be differentiated from unligated probes, either through fluorescence resonance energy transfer (FRET) or another detection method.

Slide 6:
I want to mention a couple other probe amplification methods. The Invader or Cleavase method is an isothermal method used in the CerVista HPV assay that will be shown in the next slide.

Another probe amplification method that has been proposed for clinical testing exploits Qbeta replicase. Qbeta replicase is an RNA-dependent RNA polymerase from the Qbeta bacteriophage which infects *E. coli*.

While there are many scientific publications employing this method, I am not aware of any commercial clinical tests exploiting Qbeta replicase for nucleic acid amplification.

Slide 7:
In the primary reaction: A probe and an Invader® oligo anneal to a specific DNA target sequence to generate a one-base overlapping structure. The one-base overlapping structure is created with the probe and the Invader oligo on the target. The Cleavase enzyme specifically cleaves the overlapping primary probe, releasing the 5' flap plus one nucleotide. Additional probes can bind the target and are subsequently cleaved as well.

Cleaved flaps from the primary Invader® reaction combine with a fluorescence resonance energy transfer (FRET) probe in a secondary, simultaneous cleavage reaction, generating a fluorescent signal.

The combination of two different flap sequences, FRET oligos, and fluorophores allows for single-well biplex reactions to occur.

Slide 8:
Now we move on to target amplification methods, many of which are probably more familiar because of their popularity in successful clinical test kits.

- Transcription Mediated Amplification-TMA*
- Nucleic Acid Sequenced Based Amplification-NASBA*
- Strand Displacement Amplification-SDA
- Loop-mediated Isothermal Amplification-LAMP*
- Linked Linear Amplification-LLA
- Helicase Dependent Amplification-HDA*
- Recombinase Polymerase Amplification-RPA

*will discuss in more details in the following slides

Slide 9:
The first target amplification method I would like to discuss is Transcription Mediated Amplification (TMA).

Nucleic Acid Sequenced Based Amplification (NASBA) is essentially the same as TMA. TMA and NASBA are both isothermal reactions that amplify an RNA target.

TMA is used in the Hologic assays for CT/NG, HPV, and *Trichomonas,* while NASBA is used in the Biomerieux EasyQ® HIV and enterovirus assays.

Slide 10:
In step 1, the promoter probe anneals to its RNA target. In step 2, reverse transcriptase binds to the 3' end of primer and reverse transcriptase then creates a DNA copy (cDNA) of the target RNA .

In step 4, RNAseH degrades the RNA strand of the RNA:DNA hybrid.in step 5, a second primer anneals to the ssDNA and reverse transcriptase creates a dsDNA molecule with a T7 Promoter embedded at the end. RNA polymerase initiates transcription in step 7, synthesizing 1000s of copies of RNA. Some of the newly synthesized RNA amplification products can then reenter the TMA process and serve as templates for new rounds of amplification.

Slide 11:
Like TMA and NASBA, Strand Displacement Amplification (SDA) requires multiple enzymes.

In contrast, it requires multiple primers in a specific order (a total of four) to amplify the target sequence and displace the copied sequence.

In the target generation phase, an engineered primer that has a restriction enzyme site incorporated into it binds to its complementary target and initiates strand synthesis using a thermostable polymerase. A bumper primer displaces the strand generated from the primer containing the restriction enzyme site. Because the newly generated strands incorporate thiolated dCTP, they are not susceptible to restriction enzymatic digestion. A thermostable restriction enzyme introduces a single-strand nick into the double-stranded molecules. DNA polymerase then extends the new strand and thereby displaces the strand 3’ to the nick.

Slide 12:
Loop- mediated amplification is a very elegant, yet very complex, isothermal method that utilizes DNA polymerase, and 4 primers recognizing 6 regions in the target. The reaction proceeds using strand displacement from the polymerase. This method is used in Meridian illumigene® assays and by other clinical test manufacturers as well.

The next slide shows the complexity of the structures created during LAMP. I am afraid it would take much more than our allotted time for a step-by-step discussion of this method.

A very nice description of LAMP is available from:
Norihiro T, Yasuyoshi M, Hidetoshi K & Tsugunori N. “Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products” Nature Protocols 2008; 3: 877-882.

Slide 13:
The dumbbell-like structure created from the reaction shown of the left side of this slide becomes the central molecule in STEP 1 of the LAMP cycling reaction shown on the right side.

Slide 14:
Helicase dependent amplification, as the name implies, this method utilizes DNA helicase to unwind and generate single-stranded DNA to which target-specific primers can anneal and be extended by DNA polymerase.

Because both enzymes are active at a single temperature and generation of the single-stranded target is achieved enzymatically, this is an isothermal reaction.

Quidel uses this method in conjunction with a lateral flow detection method so that the amplified product can be visualized without the need for additional instrumentation.

Slide 15:
This figure depicts the helicase and DNA polymerase enzymes interacting with DNA in the HDA reaction.

Slide 16:
Finally, I just want to touch on Whole Genome Amplification methods. These methods are target sequence independent and should amplify any and all DNA in the reaction. Again, we could devote an entire pearl presentation to any of these methods so I am afraid I will not give any of these the attention they deserve.

I am deviating from the title of this talk by including two PCR methods for WGA:

- Degenerate Oligonucleotide PCR (DOP-PCR)
- Primer Extension Preamplification (PEP)

Additionally, there are other PCR independent methods:

- Multiple Displacement Amplification (MDA)
- Multiple Annealing and Looping Based Amplification Cycles (MALBAC)

I hope that I have provided a useful overview of these methods and a taking off point for you to investigate them further.

Slide 17: References

Slide 18: Disclosures

Slide 19: Thank You from www.TraineeCouncil.org
Thank you for joining me on this Pearl of Laboratory Medicine on “Nucleic Acid Amplification: Alternatives to PCR.” My name is Tim Uphoff.