Section 2: Objectives

- Understand and identify the customary steps that occur in a single PCR amplification cycle.

- Understand and identify the essential components required for successful amplification of HLA genes by PCR.

- Understand and identify the ASHI standards that pertain to the maintenance and monitoring of the PCR environment, quality control of the thermocycler and ancillary equipment associated with the PCR process.
Section 3: Introduction

The polymerase chain reaction (PCR) was developed in the 1980s. Its first application to human genetics was the amplification of human \( \beta \)-globin DNA for the prenatal diagnosis of sickle cell anemia. After its introduction to the scientific community, PCR rapidly gained acceptance as a powerful tool for the study of a wide variety of genetic systems, including HLA.

All methods of molecular typing of HLA have a common thread: the analysis of those portions of cellular DNA which encode HLA proteins. While older methods of molecular typing required large quantities of genomic or cloned DNA, current methods utilizing PCR require only small amounts of genomic DNA which are amplified to a high copy number.

CUSTOMARY STEPS IN THE POLYMERASE CHAIN REACTION

PCR exploits the naturally occurring features of DNA chemistry and replication to produce millions of copies of a selected sequence using only a small quantity of template. The steps in a single PCR cycle proceed as follows:

1) **DENATURATION**
   Double stranded DNA is denatured by heating to 94-96°C; this breaks the non-covalent bonds between hydrogen ions on opposing bases and yields single stranded DNA. These complementary single strands serve as templates for the production of new DNA.

2) **ANNEALING**
   The denatured DNA is cooled to 50-77°C so that primers can anneal with their complementary template sequences.

3) **EXTENTION**
   Extension (replication) is catalyzed by a DNA polymerase which brings the proper deoxynucleotide triphosphates (dNTPs) into position on the polymerizing chain. The temperature and duration of this step generally range from 60-72°C for 10-60 seconds, respectively, depending on the enzyme and the stage within the amplification protocol.

After step 3 the cycle begins again. The reaction mixture is heated to denature the double stranded DNA, the temperature is lowered to allow for annealing of primer to template, and polymerization of new strands follows. The cycle of
denaturation, annealing, and extension is generally repeated 30-35 times, yielding more than $10^8$ new copies, or amplicons.

**ESSENTIAL COMPONENTS OF SUCCESSFUL PCR AMPLIFICATION**

There are seven essential factors which are required for amplification of HLA genes by PCR:

1) **DNA**, which serves as a template.
2) **Taq Polymerase**, a heat-stable enzyme which catalyzes the replication of template DNA
3) Two oligonucleotide primers, which allow initiation of replication
4) **dNTPs** (deoxynucleotide triphosphates) to incorporate into the newly synthesized DNA
5) **Buffer** to ensure optimal pH and stability for polymerase activity
6) **MgCl$_2$** to provide divalent cations required by the Taq polymerase
7) **Thermocycler** for inducing rapid temperature changes in a timed set of cycles to facilitate denaturation, annealing, and extension.

A single-stranded DNA Template serves as the code for replication of the complementary strand. It must be intact, not fragmented or degraded, or the new complementary strand cannot be synthesized properly. Most HLA laboratories utilize fresh peripheral blood anticoagulated with acid citrate dextrose (ACD) or ethylenediaminetetraacetic acid (EDTA) as the ideal source of genomic DNA; however, buccal cells from the mouth, hair roots, cultured cells, and dried blood on filter paper or cloth may also provide adequate specimen. The DNA must be free of substances that inhibit the Taq polymerase used to catalyze polymerization, such as heparin and hemoglobin. The required level of purity of the DNA often depends on the intended use of the amplified DNA: as an example, a higher level of purity is generally required for SSP typing as opposed to SSOP typing. Most manufacturers’ DNA isolation kits provide a statement as to the level of purity of DNA one can expect when utilizing these kits.

**Taq Polymerase** catalyzes the polymerization of nucleotides onto a single-stranded DNA template and is essential for successful PCR. It is a thermo-stable DNA polymerase derived from the bacterium *Thermus Aquaticus*, which thrives in hot springs. Taq’s ability to withstand high temperatures and the speed with which it is able to synthesize DNA are its most important features.

**Primers** are synthetically produced, single-stranded oligonucleotides of approximately 20-25 bases in length. They serve 2 primary functions: 1) to provide 3’ ends to which dNTPs are attached during DNA synthesis; 2) to target the double-stranded sequence to be replicated. PCR for HLA typing customarily requires a forward primer and a reverse primer – i.e., a primer that anneals to
each template strand. During the first cycle of PCR the primers anneal to their complementary genomic sequences and replication proceeds from their 3’ end: ie, nucleotides are added to their 3’ends. In subsequent cycles the newly synthesized strands as well as the original genomic DNA serve as templates. The most important position in a primer is the 3’ base, since annealing of primer and template at this position is critical for replication to commence. Other positions in the primer also provide specificity as well as stability to the annealing process, but in a polymorphic system such as HLA where alleles may differ by a single base, discrimination between these alleles for typing purposes is often dependent upon the ability of the primer to discriminate between a perfectly matched template and a template with a single mismatch.

The building blocks of DNA are the following dNTPs: deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). The purine dATP always pairs with the pyrimidine dTTP and the purine dGTP always pairs with the pyrimidine dCTP.

The buffer is responsible for maintaining proper pH during PCR. The optimal pH at which Taq Polymerase catalyzes the polymerization of new strands of DNA is 8.0-8.3. Buffer sufficient to maintain this pH is essential to successful amplification. Buffer commonly used is 10mM Tris-HCL.

The magnesium ions in MgCl$_2$ are essential for optimal catalysis by Taq polymerase. The optimal concentration may vary depending on the primers utilized, although many reactions perform well with 2.0 mM MgCl$_2$.

The first automated thermocycler was produced by Cetus Engineering in the mid 1980s. Thermocyclers have revolutionized molecular biology by providing a simple method for rapid and frequent temperature changes with minimal human intervention. Thermocyclers must be programmed with a given set of parameters for denaturation temperature and time, annealing temperature and time, extension temperature and time, and number of cycles. Additionally, the user must choose parameters specific to the type of primer being utilized.
Section 4: Methodologies / Instrumentation

Depending on the HLA DNA typing methodology utilized, PCR amplification may be specific or generic.

- In **specific** amplification, the HLA specificity is determined in the amplification step; a single allele or a group of related alleles are amplified.
- In **generic** amplification, all alleles at a particular locus or set of loci are amplified. Following amplification, these copies may be utilized in several ways to determine which alleles are present.

The three most common methods of DNA-based HLA typing are:

- **SSP (Sequence Specific Primers)** – uses specific primers. Often used in solid organ transplantation (especially for stat cadaveric organ donors), and bone marrow / stem cell transplantation.
- **SSOP (Sequence Specific Oligonucleotide Probes)** – uses generic primers. Primarily used for solid organ transplantation.
- **SBT (Sequence Based Typing)** – uses generic primers in primary amplification; generic and specific primers are used in a second round of amplification. Useful to Bone marrow / stem cell transplantation, especially in high volume laboratories.

Laboratories must choose the most appropriate methodologies for their needs based on the level of resolution required. “High” resolution refers to typing that can identify actual HLA alleles, as opposed to “low” resolution which usually defines the typing at the serological or phenotypic level.

- Bone marrow / stem cell transplantation typings must be high resolution
- Solid organ transplantation typings are generally performed at low resolution, however, in instances where the patient has an allele specific antibody, higher resolution typing of the donor and recipient may be required.

Other factors influencing decisions for testing may include cost, average turnaround time, and number of average samples per day.

Section 5: Click here to link to the web based ASHI U Procedure Manual
Section 6: Frequently Asked Questions

Question: What basic work practices should be adhered to when working in the PCR environment?

Answer: Always step on a tacky mat prior to entering a pre-amplification area to avoid bringing any potential contamination into the environment. Step on a tacky mat when exiting a post-amplification area to avoid contaminating other areas with amplicons. Change tacky mats frequently. Use dedicated lab coats, pulling gloves over lab coat cuffs, and change gloves frequently. Always decontaminate your work area prior to testing. Spin down all reagent and sample tubes prior to opening to avoid aerosols. Work with only one open tube at a time. Be sure to accurately pipette all samples and reagents using filtered pipette tips. Work should flow from pre-amplification areas to post-amplification areas. Do not take equipment, supplies, or samples from a post-amplification area to a pre-amplification area.

Question: Why is a negative control included in PCR amplification?

Answer: The negative control serves as a contamination control. It contains all the essential elements for amplification except sample DNA. No amplifiable DNA should be detected in the negative control. If DNA is detected, this invalidates the entire test, as one cannot be sure what amplified: sample DNA or some other source. Contamination of reagents, equipment, and surfaces should be investigated and resolved.

Question: What could cause a total amplification failure?

Answer: Total failure to amplify samples could be caused by a thermocycler performance or programming failure, reagent failure, or technologist error. Thermocycler: check to see that the thermocycler is programmed correctly, the correct program was selected, cycles were not interrupted and that regular function checks have been performed and are satisfactory. Reagents: Check that all reagents were added, were used in the proper amounts and concentrations, were not outside Ph range, and were not used past date of expiration. Keep a record of all reagent lot numbers so that potential problems with particular reagents may be more easily determined.

Question: What can cause a sporadic amplification failure?

Answer: If only a few test samples fail to amplify, chances are that the reagent mix was correct and complete and the thermocycler was functioning properly. This type of problem is more likely to be caused by the DNA template itself (amount, concentration, or purity). Verify the concentration and purity of the DNA sample. DNA contaminated with heparin or heme will inhibit PCR. Other common inhibitors include ETOH, bleach, and EDTA.
Section 7: Applicable ASHI Standards

The ASHI standards sited in this document are from the version noted at the top of the page. Revisions to the ASHI standards may have taken place since the development of this document. Laboratories and individuals should always reference the most current ASHI approved standards.

D.4.1.8.5 Laboratories performing nucleic acid testing must have written criteria or protocols for preventing DNA contamination using physical and/or biochemical barriers for assays involving amplification of templates.

D.5.2.2 Laboratories performing Amplification-based nucleic acid testing must:
D.5.2.2.1 Use a method to prepare DNA that provides sufficient quality (e.g., purity, concentration) and quantity to ensure reliable test results. Written protocols must specify the minimal acceptable sample in terms of volume or numbers of nucleated cells. If tests are performed without prior purification of nucleic acids, the method must be documented and validated in the laboratory.
D.5.2.2.2 Ensure that samples are stored under conditions that preserve the integrity of the nucleic acids that will be tested.
D.5.2.2.3 Ensure that template quantity and quality are sufficient to provide interpretable data for a locus (or loci) or allele(s).
D.5.2.2.4 Ensure that the amount of amplification template in each amplification reaction is in an acceptable range.
D.5.2.2.5 Ensure that aliquots of all batches of reagents (solutions containing one or multiple components) utilized in the amplification assay are demonstrated to be free of contamination.
D.5.2.2.6 Ensure that reagents used for primary amplification are not exposed to post-amplification work areas.
D.5.2.2.7 Ensure that reagents used for secondary amplification are stored in a contamination-free area.
D.5.2.2.8 Define criteria and perform quality control testing to confirm specificity for each lot and shipment of primers and probes.
D.5.2.2.9 Ensure that each lot and shipment of primers or probes is monitored to confirm stability and performance of the primers or probes.
D.5.2.2.10 Ensure that oligonucleotide probes and primers are stored under conditions that maintain specificity and sensitivity.
D.5.2.2.11 Verify that the conditions for primer extension (e.g. polymerase type, polymerase concentration, primer concentration, concentration of nucleotide triphosphates) are appropriate for the template (e.g. length of sequence, GC content).
D.5.2.2.12 Ensure that for each set of primers, conditions that influence the specificity or quantity of amplified product have been demonstrated to be satisfactory for the range of samples routinely tested.
D.5.2.2.13 Set the number of cycles at a level sufficient to detect the target nucleic acid but insufficient to detect small amounts of contaminating template.
D.5.2.2.14 Monitor the quantity of specific amplification products (e.g., gel electrophoresis, hybridization).
D.5.2.2.15 Recognize and document ambiguous combination(s) of alleles for each template/primer or template/probe combination and have procedures available to resolve these as appropriate for the clinical use of the test results.
D.5.2.2.16 Define and document the genetic designation (e.g., locus) of the target amplified by each set of primers or hybridized with probes.
D.5.2.2.17 Define the specificity and sequence of each primer and/or probe.
D.5.2.2.18 Routinely monitor for contamination of pre-amplification areas by the most common amplification products that are produced in the laboratory.
D.5.2.2.19 Routinely monitor pre-amplification work areas with wipe tests.
D.5.2.2.19.1 Monitor potential contamination using a method that is at least as sensitive as routine test methods and that uses appropriate testing primers. At least one negative (no nucleic acid) and one positive control must be included in each amplification assay.
D.5.2.2.19.2 If contamination is detected, clean the area to eliminate the contamination and document re-testing, as well as the measures taken to prevent future contamination.
D.5.2.2.19.3 Document acceptable electrophoretic conditions used for each gel electrophoresis.
D.5.2.2.20 If the size of a nucleic acid is a critical factor in the analysis of the data:
D.5.2.2.20.1 In each gel, include size markers that produce discrete electrophoretic bands spanning and flanking the entire range of expected fragment sizes.
D.5.2.2.20.2 The amount of DNA loaded in each lane must be within a range that ensures equivalent migration of DNA in all samples, including size markers.
D.5.2.2.21 Define and document the specificity and sequence of primer targets. The genetic designation (e.g. locus) of the target amplified by each set of primers must be defined and documented. For each locus analyzed, the laboratory must have documentation that includes the chromosome location, the approximate number of alleles, and the distinguishing characteristics (e.g. sizes, sequences) of the alleles that are amplified.
D.5.2.2.22 Have acceptable limits of signal intensity for positive and negative results. If these are not achieved, acceptance of the results must be justified and documented.
D.5.2.2.23 Adhere to the established criteria for accepting or rejecting an amplification assay or document the justification for acceptance of an assay when acceptance criteria are not met.
D.5.2.3 Laboratories performing SSOP methods must:
D.5.2.3.1 Define the specificity and critical polymorphic sequence of each primer and probe.
D.5.2.3.2 Label probes by a method appropriate for the testing procedure.
D.5.2.3.3 Ensure that hybridization conditions for maintaining sensitivity and specificity have been established.
D.5.2.3.4 Ensure that pre-hybridization, hybridization, and detection are carried out under empirically determined conditions of concentration and stringency that are determined by the length or composition of the probe and that achieve the defined specificity.

D.5.2.3.5 Establish criteria to determine positive or negative hybridization results for each probe using nucleotide sequences, reference DNA and/or manufacturers' QC data.

D.5.2.3.6 Ensure that each probe used gives an adequate signal, and allows detection of alleles in a heterozygous individual.

D.5.2.4 Laboratories performing SSP methods must:

D.5.2.4.1 Ensure that an internal control is included for each primer mixture that will detect technical failures and that produces a product distinguishable from the specific typing product.

D.5.2.4.2 Ensure that the amplification conditions are acceptable for the primers used.

D.5.2.4.3 Include a negative (no nucleic acid) or contamination control in each assay.

D.5.2.4.4 Ensure that primers used produce adequate amounts of amplification products to be visualized.

D.5.2.5 Laboratories performing sequencing methods must:

D.5.2.5.1 Ensure that the method for preparing sequencing templates reliably generates appropriate length sequencing templates that are free of inhibitors of subsequent reactions (e.g. residual primer extension) and free of contaminants that cause sequencing artifacts.

D.5.2.5.2 Ensure that the methods employed for preparation of sequencing templates do not alter the accuracy of the final sequence (e.g. mutations created during cloning, preferential amplification).

D.5.2.5.3 Ensure that the conditions for primer extension in cycle sequencing reactions (e.g. polymerase type, polymerase concentration, primer concentration, concentration of nucleotide triphosphates, concentration of terminators) are appropriate for the template (e.g. length of sequence, GC content).

D.5.2.5.7 Ensure the use of a scientifically and technically sound method for interpretation, acceptance, and/or rejection of sequences, especially in regions that are technically difficult (e.g. compression, ends).

D.5.2.6 Laboratories performing HLA typing must:

D.5.2.6.2 Have written criteria or protocols for:

D.5.2.6.2.1 Preparation of cells or cellular component isolations (for example, solubilized antigens and nucleic acids), as applicable to the HLA typing technique(s) performed.

D.5.2.6.2.2 Selection, quality control, and usage of all typing reagents and components.

D.5.2.6.2.4 Determining when antigen or allele redefinition and retyping are required.

D.5.3.4.1 Laboratories performing chimerism testing must:
D.5.3.4.1.2 Adjust for preferential amplification in the data analysis when using amplification-based methods.
D.5.3.4.1.3. Assess and consider the stoichiometry of the reaction when more than one locus is amplified in a single amplification reaction mixture (multiplex).
D.5.3.4.1.4.1 For systems with discrete alleles (e.g., STR) run an allele ladder concurrently with patient samples collected post-transplant.
D.5.3.4.1.4.2 For systems without discrete alleles (e.g., VNTR, RFLP), for each locus tested, amplify and analyze patient and donor samples collected pre-transplant, and/or control samples demonstrated to have similar performance characteristics (e.g., sensitivity, completion in PCR) concurrently with patient samples collected post-transplant.
D.5.3.4.1.5 Include appropriate controls for the characteristic used (e.g. size, sequence polymorphism) to distinguish donor and recipient alleles in each test.
D.5.3.4.1.6 Specify criteria for accepting or rejecting the amplification of a particular genetic locus or of an individual sample.
D.5.3.4.1.8 Document the purity obtained if processing involves isolation of cell subsets. If purity is not assessed, this must be documented on the test report.
D.5.3.4.1.9 Laboratories performing STR/VNTR testing must document, for each locus analyzed, the chromosome location, the alleles known for each locus, and the distinguishing characteristics (e.g. sizes, sequences) of the alleles that are amplified.
D.5.3.8.4 Laboratories performing parentage/relationship testing using nucleic acid analysis must:
D.5.3.8.4.4 Use a negative control to monitor for contamination, and a positive control to verify the accuracy of the procedure.
D.5.3.8.4.7 Laboratories performing STR/VNTR testing must also:
D.5.3.8.4.7.1 Use adequate electrophoretic measurement of STR/VNTR size and use ladders of known size or repeat numbers which encompass the commonly reported alleles.
D.5.3.8.4.7.2 Document, for each locus analyzed, the chromosome location, the approximate number of known alleles, and the distinguishing characteristics (e.g. sizes, sequences) of the alleles that are amplified.
D.5.3.8.4.7.3 Use ladders of known size or repeat numbers which encompass the commonly reported alleles when performing electrophoretic measurement of STR/VNTR size.
Section 8: Literature Citations and Recommended Reading

References:


Hunt, Margaret.  Real Time PCR. University of South Carolina School of Medicine, 2005.

Lind, Curt.  Principles of Molecular HLA Typing: Where we are Today.  2011 ASHI Regional Educational Workshops.