

February 2007

**Laboratory Protocol for Roche AMPLICOR®
HIV-1 DNA Testing of Dried Blood Spots**

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Introduction

Before beginning this experiment, please take the time to read and understand the handling, storage, and safety guidelines discussed in the product handbook (pages 2-8). This test is approved for research use only and is not to be used in diagnostic procedures. This protocol has been designed for qualitative testing of HIV-1 DNA using dried blood spots (DBS). For this purpose, some of the steps in the original protocol do not apply.

The AMPLICOR HIV-1 DNA Test is a qualitative *in vitro* test for the detection of HIV-1 DNA in human whole blood. The test utilizes amplification of target DNA by PCR and nucleic acid hybridization for the detection of HIV-1 DNA in human whole blood. The test is based on four major processes: sample preparation; PCR amplification of target DNA using HIV-1 specific complementary primers; hybridization of the amplified products to oligonucleotide probes specific to the target(s); and detection of the probe-bound amplified products by colorimetric determination.

The AMPLICOR HIV-1 DNA Test permits multiplexed PCR amplification of HIV-1 target and HIV-1 Internal Control DNA. The Master Mix reagent contains a biotinylated primer pair specific for both HIV-1 and HIV-1 Internal Control DNA. The detection of amplified DNA is performed using target-specific oligonucleotide probes that permit the independent identification of HIV-1 amplicon and HIV-1 Internal Control amplicon. Appendix 2 shows a workflow diagram of the major steps involved.

Sample Preparation

DNA is isolated from dried blood spots (DBS) by using the Chelex extraction method described in Appendix 1. The minimum recommended DBS size is ½ of one 6mm circle. It is important to note that a volume of at least 140µL is recommended in the final extraction and a minimum of 50µL of template DNA solution is required per sample/test. Along with the extraction of unknown sample DBS, prepare and extract a set of known HIV positive and negative control DBS to serve as controls in this experiment.

PCR Amplification

Target Selection

The AMPLICOR HIV-1 DNA Test uses the primers SK145 and SKCC1B to define a sequence of 155 nucleotides within the highly conserved region of the HIV-1 *gag* gene. The *gag* region encodes the group-specific antigens or core structural proteins of the virion. The HIV-1 *gag* genes are generally about 1500 nucleotides in length and are located at the approximate positions 789-2290 in the HIV genome. The nucleotide sequence of the primers has been optimized to yield equivalent amplification of Group M subtypes of HIV-1.

Target Amplification

PCR amplification reactions are performed with the thermostable recombinant enzyme *Thermus thermophilus* DNA Polymerase (*rTth* pol). In the presence of manganese and under the appropriate buffer conditions, *rTth* pol has DNA polymerase activity. Processed samples are added to the amplification mixture in reaction tubes in which PCR

amplification occurs. The reaction mixture is heated to denature the double-stranded DNA helix and expose the HIV-1 and Internal Control target sequences. As the mixture cools, the biotinylated primers (SK145 and SKCC1B) anneal to the HIV-1 target and HIV-1 Internal Control DNA strands. The *rTth* pol extends the primer, and a second DNA strand is synthesized. This completes the first cycle of PCR, yielding a double-stranded DNA copy of the target region of the HIV-1 and the HIV-1 Internal Control DNA. The reaction mixture is heated again to separate the resulting double-stranded DNA and expose the primer target sequences. As the mixture cools, the biotinylated primers SK145 and SKCC1B anneal to the target DNA. *rTth* pol, in the presence of excess dNTPs, extends the annealed primers along the target templates to produce a 155-base pair double-stranded DNA molecule termed an amplicon. This process is repeated for a number of cycles, each cycle effectively doubling the amount of amplicon DNA. Amplification occurs only in the region of the HIV-1 genome between the primers; the entire genome is not amplified.

Internal Control Amplification

In enzyme-based amplification processes such as PCR, efficiency can be reduced by inhibitors that may be present in the sample. The HIV-1 Internal Control has been added to the AMPLICOR HIV-1 DNA Test to permit the identification of processed samples containing substances that may interfere with PCR amplification. The HIV-1 Internal Control is a cloned DNA fragment with primer binding regions identical to those of the HIV-1 target sequence, a randomized internal sequence of similar length and base composition as the HIV-1 target sequence, and a unique probe binding region that differentiates the HIV-1 Internal Control from HIV-1 target amplicon. These features were selected to ensure equivalent amplification of the HIV-1 Internal Control and the HIV-1 target DNA. The HIV-1 Internal Control DNA is introduced into each sample with the PCR Master Mix and serves as an amplification control for each independently processed sample. Both amplifications occur in the same PCR reaction well.

Hybridization Reaction

Following PCR amplification, the HIV-1 amplicon and the HIV-1 Internal Control amplicon are chemically denatured to form single-stranded DNA by the addition of Denaturation Solution. Aliquots of denatured amplicon are added to separate wells of microwell plates (MWP) coated with either HIV-1 specific (SK102) or HIV-1 Internal Control-specific (CP35) oligonucleotide probe. The biotin-labeled HIV-1 and HIV-1 Internal Control amplicon are hybridized to the target-specific oligonucleotide probes bound to the wells of the MWP. This hybridization of amplicon to the target-specific probe increases the overall specificity of the test.

Detection Reaction

Following the hybridization reaction, the MWP is washed to remove unbound material and Avidin-Horseradish Peroxidase Conjugate is added to each well of the MWP. The Avidin-Horseradish Peroxidase Conjugate binds to the biotin-labeled amplicon hybridized by the target-specific oligonucleotide probe (HIV-1 or HIV-1 Internal Control) bound to the MWP. The MWP is washed again to remove unbound conjugate and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine

(TMB) is added to the wells. In the presence of hydrogen peroxide, the bound horseradish peroxidase catalyzes the oxidation of TMB to form a colored complex. The reaction is stopped by addition of a weak acid and the absorbance at 450 nm is measured using an automated microwell plate reader.

Experimental Procedure

PCR Preparation

1. The AMPLICOR HIV-1 DNA Test kit should be stored in its box, in the refrigerator (4°C) when not in use.
2. Begin the experiment in a lab notebook by designing a sample identification table in the standard 12 x 8 format. List all controls (no template control, HIV negative control, and HIV positive control) and samples to be tested. It is best to always duplicate the HIV positive and negative controls. The HIV negative and positive sample controls provided in the kit will not be used. Chelex extracted control DNA from DBS will be used instead. Please consider the following when arranging samples: the hybridization reaction step uses microwell plates that have removable strips of 8 wells in a standard 12 x 8 format. This allows the experiment to run on a minimum number of wells and saves the unused hybridization wells for another time. Arrange the controls and samples starting from the top left well A1 and working down the column to H1 and continuing to the next column. See the example below:

	1	2
A	No template control (water)	Sample 4
B	HIV-1 Negative control	Sample 5
C	HIV-1 Negative control	Sample 6
D	HIV-1 Positive control	Sample 7
E	HIV-1 Positive control	Sample 8
F	Sample 1	Sample 9
G	Sample 2	Sample 10
H	Sample 3	Sample 11

3. Work in a PCR hood to prepare the master mix as described accounting for 20% more than the number of samples/controls being tested. Use the table below to calculate the volumes of reagents mix in making the master mix. After calculating the volume of reagent needed, vortex each reagent and combine to make a master mix

Reagent	Stock Aliquot (µL / vial)	Volume/well (µL)	Multiple (# wells + 20%)	Volume in Master Mix (µL)
HIV-1 MMX, v1.5	700	43.75	X _____	
HIV-1 Mn ²⁺ , v1.5	200	6.25		
HIV-1 IC	100	2.0		

4. Add 52 µL of master mix to each well of a standard PCR plate that corresponds to the arrangement of controls and samples using a pipettor with aerosol barrier tips.

5. Add 50 μL of samples and controls to appropriate reaction wells using a pipettor with a new aerosol barrier tip for each sample. It is optional to add 50 μL of PCR water to the No Template Control well. Be careful to avoid cross contamination. Cap the tubes and apply pressure for a tight seal.

Amplification

1. Place the sealed PCR plate thermal cycler block.
2. Program the Applied Biosystems 96-well GeneAmp PCR System 9700 thermal cycler by creating a Method as follows:
 - HOLD Program: 3 min. at 50°C
 - CYCLE Program (5 cycles): 10 sec. at 94°C, 10 sec. at 50°C, 10 sec. at 72°C
 - CYCLE Program: (35 cycles): 10 sec. at 90°C, 10 sec. at 54°C, 10 sec. at 72°C
 - HOLD Program: 15 min. at 72°C

In the CYCLE Programs, all of the up and down ramp rates must be adjusted to 50% of the default setting of 100%. Change the ramp rates as follows - From the Create screen, select the CYCLE Program with the cursor and press the More function to access the Modify screen. Press Modify from the Modify screen to open the Select Modification screen. Press Ramp to access the Ramp Rate Modification screen. Use the circular key to select a ramp to modify and select 50% Slower. Repeat this setting for each of the up and down ramp rates in the CYCLE Programs and press Accept.

During run set-up, set the Reaction Volume to 100 μL by first changing the Ramp Speed from the 9600 Mode to the Max Mode. To do this, cursor down to Ramp Speed and select Max. Then, cursor back up to Reaction Volume and key in 100 μL .

3. Start the METHOD program. The program runs approximately 1 hour and 45minutes. The PCR plate must be removed within 15 minutes during the final HOLD program.
4. Remove the PCR plate from the thermal cycler at any time during the final HOLD program and continue immediately with Step 5. Do not allow the reaction tubes to remain in the thermal cycler beyond the end of the final HOLD program and do not extend the final HOLD program beyond 15 minutes. **DO NOT BRING AMPLIFIED SAMPLES INTO THE PCR HOOD AS AMPLIFIED CONTROLS AND SAMPLES ARE A MAJOR SOURCE OF POTENTIAL CONTAMINATION. FROM THIS POINT ON, WORKING ON A LAB BENCH IS ACCEPTABLE.**
5. Remove the caps from the reaction tubes **CAREFULLY** to avoid creating aerosols of the amplification products. Immediately pipet 100 μL **[1] DN** to the first column (or row) of reaction tubes using a multichannel pipettor with aerosol barrier tips and mix by pipetting up and down. For each column (or row), repeat this procedure using a fresh set of tips. Incubate for 10 minutes at room temperature to allow complete denaturation.

6. The denatured amplicon can be held at room temperature for no more than 2 hours before proceeding to Detection. If the detection reaction can not be performed within 2 hours, cap the tubes with new caps and store the denatured amplicon at 2-8°C for up to one week.

Detection

1. Warm all reagents to room temperature.
2. Prepare Working Wash Solution as follows. Examine **10X WB**, and if necessary, warm at 30-37°C to redissolve any precipitate. Add 90mL **10X WB** to 900mL of distilled or deionized water. Mix well. For washing, prepare 40 mL of Working Wash Solution for each 8-well MWP strip. Working Wash Solution should be stored at 2-25°C in a clean, closed plastic or glass container and is stable for 2 weeks from the date of preparation.
3. Allow **HIV-1 MWP** and **HIV-1 CT MWP** to warm to room temperature before removing from their foil pouch. Remove the appropriate number of 8-well MWP strips from the foil package(s) and set into the white MWP frame. Use tape to secure the strips to the frame and label the columns. Return unused strips to pouch and reseal making sure that the desiccant remains in the pouch. NOTE: MWP strips must be handled carefully to avoid breakage. To remove strips from the frame, pry the top and bottom tabs. The frame can later be reused to hold strips in additional experiments.
4. Add 100 µL [**2**] **HIV-1 HYB** to each well on the MWP to be tested.
5. If the denatured amplicon were stored at 2-8°C, incubate at 37°C for 2-4 minutes in order to reduce viscosity.
6. Using aerosol barrier tips, pipet 25 µL of denatured amplicon to the appropriate well(s) of the both Test and CT MWP. Gently tap the plate approximately 10-15 times until the color changes from blue to light yellow (this color change indicates sufficient mixing has occurred).
7. Cover the MWP with MWP lid; incubate for 1 hour at 37°C ± 2°C.
8. Wash the MWP 5 times manually using the Working Wash Solution.
 - a. Empty contents of plate and tap dry on paper towels.
 - b. Pipet Working Wash Solution to fill each well to top (250-300 µL). Let soak for 30 seconds. Empty out contents and tap dry.
 - c. Repeat Step (b) 4 additional times.
9. Add 100 µL [**3**] **AV-HRP** to each well. Cover the MWP and incubate for 15 minutes at 37°C ± 2°C.

10. Prepare Working Substrate by mixing 2.0 mL **[4A] SUB A** and 0.5 mL **[4B] SUB B** for each multiple of two, 8-well microwell strips. Prepare Working Substrate no more than 3 hours before use. Store at room temperature and protect from exposure to direct light.
11. Wash the MWP as described in Step 8.
12. Add 100 μ L of Working Substrate into each well being tested using a multichannel pipettor.
13. Allow color to develop for 10 minutes at room temperature (20-25°C) in the dark.
14. Add 100 μ L **[5] STOP** to each well using a multichannel pipettor.
15. Measure the absorbance at 450 nm within 30 minutes of adding **[5] STOP**. Record the absorbance value for each sample and control tested.

Quality Control

At least one duplicate of the HIV-1 negative control and one duplicate of the HIV-1 positive control must be included in each test run. As with any new laboratory procedure, new operators should consider the use of additional positive and negative controls each time the test is performed until such time that a high degree of confidence is reached in their ability to perform the test procedure correctly.

Negative Control

The assay result for the HIV-1 negative control should be less than 0.2 A_{450} . If the HIV-1 negative control value is greater than or equal to 0.2 A_{450} , the run should be invalidated and the entire test procedure (PCR preparation, amplification, and detection) should be repeated. If the absorbance value for the HIV-1 negative control is consistently greater than 0.2 A_{450} , repeat Chelex extraction on a different DBS that is HIV-1 negative.

Positive Control

The assay result for the HIV-1 positive control should be greater than or equal to 1.5 A_{450} . If the HIV-1 positive control value is less than 1.5 A_{450} , the run should be invalidated and the entire test procedure (PCR preparation, amplification, and detection) should be repeated. If the absorbance value for the HIV-1 positive control is consistently less than 0.2 A_{450} , repeat Chelex extraction on a different DBS that is HIV-1 positive.

Results

For a valid run, sample results are interpreted as follows:

HIV-1 MWP A_{450}	IC MWP A_{450}	Interpretation
< 0.2	≥ 0.2	HIV-1 DNA Not Detected. Sample is presumptive negative for HIV-1 DNA
< 0.2	< 0.2	Inhibitory Sample. HIV-1 DNA, if present, would not be detectable. Process another aliquot of the original sample and repeat the test. Inhibitors are often labile and samples initially inhibitory may not be inhibited when repeated. If the original sample is not available, a new sample must be collected.
≥ 0.8	ANY	HIV-1 DNA detected. Sample is positive for the presence of HIV-1 DNA.
$\geq 0.2, < 0.8$	ANY	Equivocal. Results are inconclusive for HIV-1 DNA. Repeat testing must be performed on the processed sample regardless of the IC result. The final interpretation of these specimens should be determined using 0.2 A_{450} as the cutoff, as indicated in the table below.

Interpretation of repeat testing:

Repeat HIV-1 MWP A_{450}	Repeat IC MWP A_{450}	Interpretation
At least 1 result ≥ 0.2	ANY	HIV-1 DNA detected. Sample is positive for the presence of HIV-1 DNA.
Both results < 0.2	Both results ≥ 0.2	Sample is presumptive negative for HIV-1 DNA
Both results < 0.2	At least 1 result < 0.2	Invalid Result

Clean-Up

Completed MWP's can no longer be read after 30 minutes and should be discarded. Remove the strips from their white frames, discard the strips, and save the frames for the next set of MWP strips. All extra reagents should be kept refrigerated until all MWP strips are used. After all MWP strips are used, all remaining reagents can be disposed following the safety guidelines in the product handbook. Each new kit comes with 30% extra reagent to account for pipetting and repeats.

Appendix 1

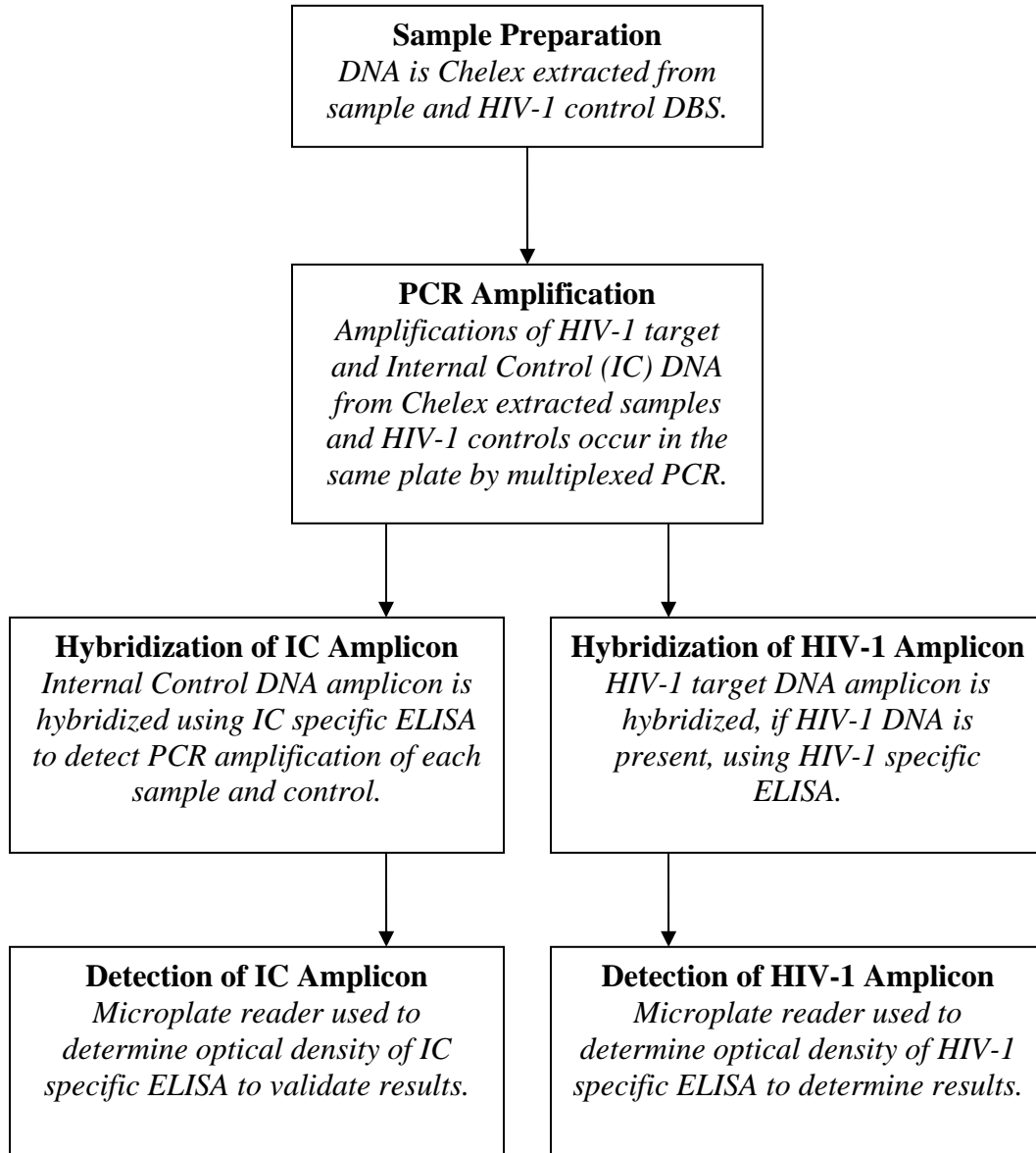
Chelex DNA Extraction

Materials: Dried, blood-blotted filter papers; 10% saponin in water (stored at -20°C); 1XPBS (Calcium and Magnesium Free pH7.4); 20% Chelex (stored at room temperature); a heat block at 95°C

1. Cut the filter paper to appropriate size using a scissors or hole puncher. The same blade or hole puncher can be used after wiping with an alcohol swab.
2. Combine the dried, blood blotted filter papers with 1ml of PBS and 50ul of 10% saponin in a 1.5 ml Microfuge tube, invert several times, and store over night at 4°C.
3. Microfuge tubes for 5 seconds and aspirate the now reddish PBS/saponin from the tubes with a clean non-barrier tip attached to a Pasteur pipette at the end of a vacuum assembly using a new tip for each tube.
4. Add 1 ml of PBS/tube (no saponin), invert several times and incubate at 4 °C for 15-30min. Turn on the heat block at this time to allow time for 95 °C to be reached.
5. Microfuge and aspirate (as above) as much fluid as possible and afterwards use the tip to press the filter paper down into the lower third of the tube without packing it excessively.
6. Transfer 1ml of vortexed chelex stock solution to a 1.5 ml microfuge tube and using a tip with tapered end cut off. Dispense 50 ul to each sample, vortexing or inverting the tube every two or three transfers (to be sure you are not just transferring water with all the chelex settled to the bottom of the dispensing tube).
7. Add 100ul of sterile water to each tube.
8. Extract the DNA by incubating the tubes for 10 min in a 95 °C heat-block, vigorously vortexing each sample every 2 minutes or so throughout the incubation. It is best to briefly uncap each tube every two minutes or so in the block to release pressure, or else the tubes will "pop".
9. After incubation, microfuge the tubes for 5 minutes at high speed. Meanwhile, label two sets of 0.6mL microfuge tubes for transfer, the second set for final storage of the extracted DNA samples.
10. Transfer as much solution as possible from the spun tubes to the first set of microtubes with a 200uL barrier-tip, not worrying if chelex is carried over as well.
11. Spin tubes for 10 minutes at high speed and then transfer the final, white-to-yellowish supernatant (avoiding the pelleted chelex) to the final set of labeled tubes. Store at -20C.

Appendix 2

Workflow Diagram



Appendix 3

Minimum disposable supply requirements for planning purposes.

The following list is based on completing one test of 96 wells.

Chelex Extraction

2 boxes non barrier pipet tips for aspiration
2 boxes 200uL barrier pipet tips for transfer
1.5mL tubes for all DBS being extracted
0.6mL tubes, 2 times the number of DBS being extracted

PCR Preparation

1 box 200uL barrier pipet tips for adding sample
1 sterile trough for mixing master mix
Various barrier pipet tips for preparing and aliquoting master mix
1 96-well PCR plate
8 strips of 12 PCR caps

Detection

5-7 boxes 200uL barrier pipet tips for adding reagents
7 sterile trough for mixing master mix
4 adhesive plate covers