HIV-1 PROVIRAL POLYMERASE CHAIN REACTION (HIV-1 DNA PCR)

Using the Roche Amplicor HIV-1 Test

1. CLINCAL SIGNIFICANCE:

HIV infection is usually documented by detection of HIV specific antibodies in serum. However, serologic assays do not readily identify HIV infection in neonates with passively acquired maternal antibodies or individuals with “indeterminate” antibody profiles or early infection. These situations require follow-up serologic testing, antigen detection and/or culture.

Gene amplification techniques, e.g., Polymerase Chain Reaction (PCR), allow the detection of rare DNA sequences. PCR can detect low copy numbers (1-5) of HIV-1 proviral DNA in infected cells. PCR may help document **HIV-1** infection when routine diagnostic assays are not adequate.

The Roche Amplicor HIV-1 assay was optimized for the detection of HIV-1 subtype B DNA. The assay has reduced amplification and subsequent detection for all other subtypes. Test results must be interpreted carefully, considering the virus subtype, the virus concentration, and the sensitivity of the assay.

2. PRINCIPLE:

Characteristic of all retrovirus replication cycles, HIV single stranded RNA is transcribed into double stranded DNA (“provirus”) and integrated into the host cell genome. This integrated HIV provirus can be detected by amplification of a specific sequence in the highly conserved *gag* region of the genome.

Whole blood is treated with the Specimen Wash Solution reagent, which selectively lyses the red blood cells. The intact leukocytes are pelleted and washed. Alternatively, pellets can be prepared from the peripheral blood mononuclear cells (PBMCs) isolated using lymphocyte separation techniques described in the ACTG Specimen Processing Guidelines Protocol available on the ACTG web site. The pellets can be stored at -70°C or be treated with the Extraction Reagent (nonionic detergents, magnesium and proteinase K) to extract the DNA for analysis.
The double stranded DNA is denatured by heat to expose the target region to labeled primers. The amplification target, a highly conserved region of the *gag* genome, is bordered by the primer pair SK 431/462. Two biotinylated oligonucleotide primers complementary to the amplification target sequence will bind to the target region on the DNA. Taq polymerase links deoxynucleotide triphosphates, (dATP, dGTP, dCTP, dUTP) extending in the 5’ to 3’ direction to produce biotinylated complementary DNA sequences called “amplicons”. During the polymerase chain reaction, controlled fluctuations in temperature allow repeated denaturation, annealing and extension processes resulting in a geometric increase in the target sequences. DNA copies from previous cycles become templates in subsequent amplification periods.

Uracil-N-Glycosylase (UNG) is used to prevent contamination from previous PCR reactions. UNG will excise any dUTP found in previously amplified DNA. (Naturally occurring DNA will contain dTTP.) Before the PCR reaction is initiated, the thermal cycler is set at 55°C to optimally activate UNG for two minutes. Amplification cycles temperatures oscillate with cycles of 95°C : 55°C : 72°C and cycles of 60°C : 72°C.

After the amplification cycles are completed, the UNG is inactivated by a denaturing solution containing sodium hydroxide.

The amplified DNA is incubated in polystyrene wells containing immobilized BSA-conjugated probe (SK 102) which is specific to the biotinylated amplicons. After incubating for one hour, the unbound reactants are washed away. An avidin-horseradish peroxidase conjugate is added and incubated for 15 minutes at 37° ± 2°C. Unbound reactants are washed away. A chromogenic substrate, tetramethylbenzidine (TMB), is added for a 10 minute incubation. The reaction is stopped by the addition of H_2SO_4 and the absorbance is read at 450 nm. A fixed O.D. cutoff of 0.350 is used to determine whether a specimen is positive or negative.

Specimens may be further analyzed for the presence of HLA DQα DNA (optional assay detailed herein) to confirm the adequacy of the specimen and validate a negative DNA PCR result.

3. SPECIMENS:

3.1 Acceptable specimens include 3 to 5mLs whole blood for adults, or 0.5mL recommended for infants (with an absolute minimum of 0.1mL whole blood for infants). Blood should be collected in ACD (yellow top) or in EDTA (lavender top) vacutainers.

3.2 Dry Cell Pellets, processed and frozen according to the ACTG Specimen Processing Guidelines, are also appropriate samples for this assay.

3.3 Viably cryopreserved cells may also be used, if washed to remove the DMSO.

3.4 Heparinized (green top) blood is NOT acceptable for this assay.

3.5 Technically, other body fluids and tissues may be processed for HIV PCR. However, the HIV PCR assay has been standardized using peripheral blood specimens.

3.6 Store whole blood at 2-25°C for up to 4 days. (Do not freeze specimens prior to the white blood cell separation. The subsequent cell lysis will release the DNA, rendering the specimen inadequate, and will release hemoglobin which interferes with the PCR reaction.)

3.7 Stability parameters for other body fluids/cells have not yet been determined and validated. The current recommendation is to prepare cell pellets from other body fluids as quickly as possible - within 6 hours after collection.

4. QA POLICIES and PROCEDURAL HINTS:

4.1 At each step, specimen labeling and handling is optimized to prevent mix up or contamination. To avoid contamination of specimens and reagents:

- 4.1.1 No aliquot is ever returned to the original container.
- 4.1.2 Only one tube or container is opened at a time.
- 4.1.3 Aerosol resistant tips must be used at every step for all pipettors
- 4.1.4 Use different tips or pipets for each reagent or specimen.
- 4.1.5 Do not insert pipettor beyond the disposable tip into any container.

4.2 Work and equipment should be separated into isolated work areas, maintaining a unidirectional work flow (Pre-amplification Area One for reagent preparation; Pre-

amplification Area Two for sample and control preparation; and finally Post-amplification Area Three for amplification and detection). Amplified DNA must not be carried into either the Reagent Preparation work area or the Sample Processing area.

4.3 Powdered gloves should not be used. Powder granules may inhibit the PCR reaction.

4.4 A small amount of sand (depth of 0.5 cm) may be kept in each well of the heating blocks to ensure an even transfer of heat to the conical microtubes.

5. HIV PCR EQUIPMENT AND MATERIALS:

5.1 Pre-amplification area one—Reagent Preparation Work area:

PE MicroAmp Consumables: tubes, caps, base, tray and retainer
Repeat pipettor
1.25mL combitips, individually wrapped
Micropipettors (50uL and 100uL)
Aerosol resistant barrier pipettor tips

5.2 Pre-amplification area two—Sample Processing / Extraction Work area:

40-200µL pipettor
200-1000µL pipettor (2)
table top microfuge
1.8mL sterile microtubes
12 x 4 microtube rack
2 heating blocks
(note: waterbath or incubator may substitute for the 60°C unit)
3mL sterile plastic disposable fine-tipped transfer pipets
Aerosol resistant barrier pipettor tips

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5.3 Post-amplification—Amplification Reaction and Product detection Work areas (may be combined):

PE thermal cycler, 9600
Multi-channel pipettor (25 & 100µL)
Matrix electronic pipettor
Microwell plate lid
Plate reader & printer
Aerosol resistant barrier pipettor tips
Disposable reagent reservoirs
incubator (37 +/- 2°C)
plate washer
LDMS, direct or data transfer via diskette

6. HIV PCR Reagents:

Roche - HIV PCR Amplicor Detect kits, listed below:

6.1 Whole Blood Specimen Kit (Roche # 87253):
H_2O, sodium phosphate, detergent, sodium azide

Extraction reagent (H_2O, Tris-HCl, MgCl2, proteinase K, detergent, sodium azide)

6.2 HIV-1 Amplification Kit (Roche #87260):

HIV-1 Positive Control (HIV-1 specific, non-infectious DNA; H_2O; Tris-HCl; EDTA; sodium azide)
HIV-1 Negative Control (non specific, non-infectious DNA; H_2O; Tris-HCl; EDTA; sodium azide)
HIV-1 Master Mix (H_2O; Tris-HCl; glycerol; KCl; dNTPs; SK431 and SK462 primers, AmpliTaq ^{R};sodium azide)
HIV-1 AmpErase (H_2O; Tris-HCl; EDTA; DTT; NaCl; solubilizer; UNG; glycerol; sodium azide)

6.3 HIV-1 Detection Kit (Roche # 87271):

Wash Concentrate (H_2O; NaCl; sodium phosphate; detergent; Proclin 300 ^{TM} as a preservative))
PCR Denaturation Solution (NaOH; EDTA; H_2O; thymol blue)
PCR Hybridization Buffer (Solubilizer; sodium thiocyanate, Sodium Phosphate; H_2O)
PCR Conjugate (TRIS-HCl; Avidin-HRPO; H_2O; Proclin 150; emulsifier; bovine gamma globulin; phenol)
PCR Substrate A (H_2O_2; Citric Acid; H_2O; Proclin 150)
PCR Substrate B (DMF; H_2O; TMB)
Stopping Reagent (Sulfuric Acid, H_2O)
HIV-1 MWP (HIV-1 DNA probe coated microwell plate, 96 well)

7. HIV DNA PCR PROCEDURE:

7.1 Sample Processing:

Location: Separate Set-Up Area with a P2 Biological Safety Cabinet
Note: Proper personal protective equipment should be used.

7.1.1 Prepare a tube for each control, VQA blinded pellet and patient specimen: pipet 1.0mL specimen wash buffer into 1.8mL Sarstedt microtubes. Label the side of each tube with the accession number or other unique identifier.

7.1.2 Each ACTG patient sample is run in duplicate.

7.1.3 Invert blood specimen in vacutainer tube* 15-20 times to mix thoroughly. Add 500µL whole blood to labeled microtube containing 1.0mL specimen wash buffer. For pediatric patient (less than 18 months old) specimens, use 100µL whole blood.
*Note: Whole blood can be stored in ACD or EDTA vacutainer tubes up to 4 days at 2-25°C prior to extraction.

7.1.4 Seal vial and mix by inversion 10- 15 times.

7.1.5 Allow mixture to stand at room temperature for 5 minutes. Vortex specimen vial thoroughly for a minimum of 15-30 seconds. Incubate specimen at room temperature for an additional 5 minutes. Vortex specimen thoroughly for a minimum of 15 - 30 seconds.

7.1.6 Microcentrifuge vial for 3 minutes at high speed (maximum 16,000xg) at room temperature.
Aspirate supernatant carefully to avoid disturbing the pellet. Add 1.0mL specimen wash buffer to vial. Vortex vial specimen thoroughly for 30 seconds. Centrifuge vial for 3 minutes at high speed (maximum 16,000xg) at room temperature.

7.1.7 Repeat Step 7.1.6. If RBCs are still detectable, repeat step 7.1.6 again.

7.1.8 Aspirate supernatant being careful to avoid disturbing the pellet. Store dry pellet at -70°C until ready to extract.

7.1.9 Note: Ficoll-derived dry cell pellets (1,000,000 cells) may also be used for this assay. (If there is any possibility the ficoll-derived pellet had contact with DMSO, as in cryopreservative media, the pellet should be washed 2x with Specimen Wash buffer before extraction. See Extraction notes at 7.3.3.)

7.2 Template & Reagent Preparation (Reconstitution of 2x Master Mix-Amperase):
Location: designated "Clean" Pre-PCR Area/ work station.
Note: Proper personal protective equipment should be used.

7.2.1 Prepare an LDMS template designating the location of the VQA controls, VQA blinded real-time controls and patient specimens. At lab discretion, intersperse the kit negative controls throughout the patient samples and add the kit positive control.

7.2.2 Add 100µL of Amperase into one vial of Master Mix. Invert mixture several 10-15 times. Do NOT vortex. This mixture is sufficient for 32 amplifications. Write the date of the Master Mix-Amperase preparation onto the vial. This mixture is stable for 4 weeks at 2-8°C.

7.2.3 Place the PCR tubes into the sample retainer tray. Aliquot 50µL of the working master mix into each PCR tube. Cover the tubes loosely with caps. Transfer to the designated *Specimen Preparation* Workstation. Store the sample tray at 2-8°C until ready to add the specimens.

7.3 Extraction:
Location: Set-Up Area
Note: Proper personal protective equipment should be used.

7.3.1 Check that the 2 separate heating blocks are heated to 60°C and 100°C (+/- 2°C). A 60°C waterbath or incubator may be substituted for the 60°C heating block.

7.3.2 For each run include the VQA controls, VQA blinded real-time controls, and 3 kit negative controls (maximum number for a 96 well run) and at least 1 kit positive control. (Kit controls have been pre-diluted with

extraction buffer.) See VQA DNA SOP for further information (http://aactg.s3.com/pub/download/vir/SOPforDNAPCRassays.doc) .

7.3.3 Remove cell pellet from -70°C. To each pellet and VQA controls, add 200 µL of extraction buffer, vortex for 15-30 seconds. Quick spin for a few seconds and then incubate at 60°C for 30 minutes.
(Note: If a ficoll-derived dry cell pellet (1,000,000 cells) is used for this assay, add 400µL extraction buffer per million cells. If the cells were cryopreserved in DMSO, wash twice in Specimen Wash solution before extracting.)

7.3.4 Immediately incubate extract at 100°C for 30 minutes. Proceed directly to PCR amplification.

7.4 Amplification Reactions:
Location: "Clean" Pre-PCR area in a designated Specimen Preparation work station.

Note: Proper personal protective equipment should be used.

7.4.1 Vortex the specimens.

7.4.2 Aliquot 50µL of the extracted sample DNA into the master mix of the appropriate tube. Verify the specimen identification against the LDMS template map location and add samples accordingly. Use positive displacement pipettors and a separate sterile aerosol resistant barrier pipet tip for each specimen and control. Use extreme care to avoid carry-over contamination and aerosols. Loosely cap each strip of PCR tubes after loading the samples. After all tubes have been loaded and capped, use the Roche "Cap Installing Tool" to firmly press caps on the microtubes.

7.4.3 Remove tubes from the retainer tray base. Carry PCR tubes in the retainer tray directly to the product workstation. Place retainer tray into the thermal cycler, aligning the notch on the retainer tray with the notch on the unit. Close the cover and turn the cover knob until the white portions of the lid & knob are aligned.

The HIV-1 proviral PCR amplification requires local definition of a Method program in the thermal cycler which links the following file programs:

Hold:	50°C,	2 minutes
5 Cycles of:	95°C,	10 seconds
	55°C,	10 seconds
	72°C,	10 seconds
30 Cycles of:	90°C,	10 seconds
	60°C,	10 seconds
	72°C,	10 seconds

Hold:	72°C,	5 minutes
Hold:	72°C	"forever"

HIV-1 DNA linked programs require approximately 75 minutes to complete.

As soon as PCR cycles are completed, press "Stop". Thermal cycler temperature will ramp down to 25°C. Immediately remove the amplified DNA from the thermal cycler.

7.4.4 Use a narrow spatula to loosen the caps. Remove caps very carefully to avoid aerosol spray. Immediately add 100µl amplicon denaturation solution to each sample, using the pre-programmed Matrix electronic pipettor (program 1). Let denatured amplicons stand at room temperature for 10 minutes before proceeding to microtiter plate hybridization assay.

7.5 Detection (microtiter plate hybridization assay for detection of PCR amplified HIV1 sequences):
Location: PCR Product Detection workstation
Note: Proper personal protective equipment should be used.

7.5.1 Allow all the reagents to come to room temperature. Remove the appropriate number of microtiter strips from the foil pack and place onto the plate holder. Add 100µL of hybridization/neutralization buffer to each well using the pre-programmed Matrix pipettor (program 2).

7.5.2 Add 25µL denatured amplicon to corresponding duplicate wells containing neutralization/hybridization buffer, using the Matrix electronic pipettor (program 3). Neutralization of the amplicons will be evident when the color changes from blue to yellow.

7.5.3 Prepare working wash solution by adding 1 volume of wash concentrate (10x) to 9 volumes of distilled or deionized water. Mix well. After the incubation, wash the plate 5 times using a microwell plate washer.
For automated washing, program the washer to:

a) aspirate contents of wells.
b) fill each well to top with working wash solution (~ 350-450 µl), soak for 30 seconds and aspirate dry.
c) repeat step (b) 4 additional times.

After washing, invert and tap the plate dry on a paper towel.

7.5.4 Add 100µL of Avidin-HRPO conjugate into each well using Matrix pipettor program 2. Cover plate with plate cover and incubate at 37°C (+/- 2°C) for 15 minutes.

7.5.5 Wash microtiter plate as in step 7.5.3. Blot plate on paper towels to remove excess solution.

7.5.6 Prepare TMB reagent just prior to use. For 12 strips (full plate), mix the following:

12mLs PCR Chromagen Reagent A + 3mLs PCR Chromagen Reagent B

(ratio = 4 parts Chromagen Reagent A : 1 part Chromagen Reagent B)

Add 100µL TMB reagent into each well using the Matrix electronic pipettor (program 2).

Immediately incubate for 10 minutes at room temperature in the dark.

7.5.7 Stop reaction with 100µL stop solution (Matrix electronic pipettor, program 2)

7.5.8 Read microtiter plate at 450 nm. Reading must be completed within one hour of adding the Stop Reagent. Results should be transmitted to the LDMS, either directly or using the remote read option. The LDMS will indicate the validity of the assay based on the VQA copy number controls and the VQA blinded real time control results.

8. RESULTS

8.1 Absorbances 0.350 or greater are considered positive;
Result = "HIV-1 DNA detected".

8.2 Absorbances below 0.350 are considered negative for HIV-1, subtype B;
Result = "HIV-1 DNA not detected".

Note that this assay has been optimized for HIV-1, subtype B. Other subtypes may yield a false negative result. All negative results must be interpreted considering the virus subtype, the concentration/collection practice, and the presence or absence of possible inhibitors. Use of the HLA DQα supplemental detection can indicate the presence of amplification inhibitors, inadequate specimen collection and processing problems. The HLA DQα detection assay will not control for the Amplicor assay's reduced sensitivity for some non-clade B viruses.

8.3 Negative results may be validated using a supplemental assay such as the HLA DQα (procedure following) to document adequacy of the specimen. (See 8.2 above)

8.4 ACTG specimens are run in duplicate. If these duplicates have discordant results, the specimens will normally need to be retested.

9. CRITERIA FOR A VALID RUN

9.1 The VQA controls* must react as expected:

* 20+ copy control should have the A_{450} reading 2.0 or greater (preferably 3.0).

* 10+ copy control should have the A_{450} reading **>**0.350.
* 5+ copy control is a +/- marker and should be positive 95% of the time. A_{450} should be greater than 0.350.
* 0 copy control must be negative. A_{450} reading must be less than 0.350.

9.2 The VQA blinded real time controls must react as expected. Expected results are preprogrammed into the LDMS database. The LDMS will indicate if the results for these unknowns are valid. Two consecutive invalid runs, or three invalid runs out of a series of ten runs, will lock the LDMS for this procedure. The lab must contact the VQA (312-942-5954 or vqa@rush.edu) for assistance to unlock the LDMS.

9.3 Kit negative control must be negative.

9.4 Kit Positive control must be positive.

9.5 Any deviation from these expected results must be reviewed with the director or designee. A run with one or more controls out of range will need to be repeated.

9.6 See Section 11 for Troubleshooting suggestions.

10. HLA DQα VALIDATION (optional assay to validate negative results)

10.1 HLA DQα Equipment:

10.1.1 Set-Up:

0.5 - 10µL pipettor	Eppendorf repeat pipettor
10-50µL pipettor	1.25mL sterile Eppendorf repeat tips
40-200µL pipettor	500µL snap cap microfuge tubes
200-1000µL pipettor	1.5mL snap cap microfuge tubes
Aerosol resistant barrier pipet tips	

10.1.2 Amplification:
Table Top Microfuge
Thermal Cycler

10.1.3 Agarose Gel Preparation:

Weigh boats	Brinkman pipet bulb
Weigh balance	10 & 50mL graduated pipets
250mL Erlenmeyer flask	Microwave

10.1.4 Sample Preparation and Gel Loading:

0.5 - 10µL pipettor	Aerosol resistant barrier pipet tips
10-50µL pipettor	Eppendorf repeat pipettor
40-200µL pipettor	1.25mL repeater tips

200-1000µL pipettor	Round bottom microtiter plate
Mini Submarine Gel	Electrophoresis power supply

10.1.5 Photography:

Polaroid Camera
Polaroid Polapan 400 Film
UV Transilluminator
---or---
Gel Documentation System, linked to a computer and printer.

10.2 HLA DQα Reagents

10.2.1 Set-Up Reagents

10.2.1.1 Master Mix:
10µL 10x TAQ buffer
20 pMoles of GH 26 *
20 pMoles of GH 27**
20 nMoles of each dNTP (dGTP, dCTP, dATP, dTTP)
0.5µl (2.5 units) of TAQ polymerase
QS to 50µL with sterile dH_2O

* GH 26 sequence: GTGCTGCAGGTGTAAACTTGTACCAG MW: 12,596.2
** GH 27 sequence: CACGGATCCGGTAGCAGCGGTAGAGTTG MW: 13,540.6

10.2.1.2 10x TAQ Buffer:
500mM KCl
100mM TRIS, pH 8.4
25mM $MgCl_2$
0.1% Gelatin

10.2.2 Detection Reagents:

10.2.2.1 TBE (Tris Boric acid EDTA solution) may be purchased commercially (Genemate Cat# C-5555-40L), or prepared locally as listed below:

5x TBE (20 liters):	10x TBE (1 liter):
1078g TRIS base	107.8g TRIS
550.3g Boric Acid	55.03g Boric Acid
93.15g EDTA	9.305g EDTA
QS to 20 liters with distilled H_2O.	QS to 1L with dH_2O.

Mix on magnetic stirrer overnight at room temperature.

Store at room temperature for 3 months.

1x TBE – Running Buffer: Dilute out 5x TBE for running buffer TBE
320mLs 5x TBE
1280mLs dH_2O

10.2.2.2 ØX DNA Ladder:
1µL 100bp DNA Ladder (BioLine HyperLadder, cat. # BIO33031)

Store at 4°C for 3 months

10.2.2.3 5x Sample Loading Buffer (BioLine, cat. # BIO37045)

10.2.2.4 Syber Green Nucleic Acid Stain (Molecular Probes, cat. # S7567)
(10,000x concentrate in DMSO)

Store at -20°C. Protect from light. Follow manufacturer's outdate.

10.2.3 Reference: General calculation for reagent preparation from stock solutions:

(example) $C_1V_1 = C_2V_2$
$5x = 1600$
$x = 320$

10.3 Extraction:
Location: Clean Set-Up Area
Note: Proper personal protective equipment should be used.

10.3.1 This assay uses the extracted patient samples and VQA controls prepared in section 7.3. (The HLA assay is controlling for the adequacy of those samples and their DNA extractions.) Two different amplifications and detections are performed because the HLA requires it's own mastermix for amplification and is detected by visualization in an electrophoretic gel.

10.3.2 Include a minimum of 3 HLA controls, containing $2x10^6$, $4x10^6$, and $6x10^6$ cells for each run. These controls should have been extracted along with the patient samples for the HIV DNA PCR assay—details in section 7.3.

10.4 Set-Up for amplification:
Location: "Clean Pre-PCR Area" designated workstation.
Note: Proper personal protective equipment should be used.

10.4.1 Include the 3 HLA controls and a negative control (dH_2O) for each run.

10.4.2 Prepare sufficient HLA Master mix solution for each of the patient samples and lab controls plus extra volume for pipetting by adding 2 to the total number of specimens in the master mix calculation below. Prepare a worksheet listing the specimen number and patient identifiers which correspond to the HIV DNA PCR worksheet.

10.4.3 Master Mix calculations and formula:

n = number of specimens and controls

n + 2 = A *(Example: 13 specimens + 2 = 15)*

(A)(50) = B
(n)(50) = Y
P, Q = concentration factors

10x TAQ Buffer	A (10)µL	15(10) =150µL
dH_2O	B - (sum of reagents excluding oil) µl	750-321 = 429µL
dNTP	A (10)µL	15*(10) =150µL*
GH 26 (P)	(A)(P)µL	15 (0.4) = 6µL
GH 27 (Q)	(A)(Q)µL	*15 (0.5) = 7.5µL*
TAQ Polymerase	(A)(0.5)µL	*15 (0.5) = 7.5µL*
Light Mineral Oil	(n)(50)µL	*13 (50) = 650µL*

10.4.4 Label 0.5mL snap cap microfuge tubes with number corresponding to specimen or control.

10.4.5 Place 50µL of light mineral oil into each 500µL specimen tube.

10.4.6 Add 50µL of master mix into each 500µL specimen tube.

10.4.7 Add 50µL of each extracted sample into its respectively labeled specimen tube.

10.4.8 Carry the labeled 500µL specimen tubes to the thermal cycler in the Product amplification workstation .

10.5 Amplification
Location: Product Amplification Workstation
Note: Proper personal protective equipment should be used.

10.5.1 Briefly centrifuge specimen tubes in a table top microfuge to ensure that the oil overlay is on top of the reaction mixture.

10.5.2 Place tubes into the Perkin-Elmer Thermal Cycler.

10.5.3 Begin the HLA DQα PCR with the predefined Method Program linking the following file programs:

HLA DQα PCR Program sequence:

30 cycles of:

95°C, 30 seconds

55°C, 30 second

72°C, 1 minute

1 cycle of:

72°C, 9 minutes

1 cycle of:

15°C, 10 minutes (optional)

Hold:

15°C, forever

The HLA DQα amplification method program requires about 1.5 hours.

10.5.4 Thirty minutes prior to completion of the HLA DQα amplification, prepare the agarose gel.

10.6 Agarose Gel Preparation:

10.6.1 Assemble the horizontal submarine gel platform with combs in place on the platform.

10.6.2 Prepare a 2% Agarose gel solution using Ultrapure electrophoresis-grade agarose. Measure into a 250mL Erlenmeyer flask:

2.0g Agarose
10mL 10X TBE
90mL ddH_2O (for a final concentration of 1X TBE)

10.6.3 Melt the agarose solution by heating in a microwave for 3 minutes. (Use a thermal protector glove to remove the agarose from the oven.) Do not shake or stir the melted agarose. Wait until the melted agarose is cooled just enough to handle the flask without the thermal gloves. Do not allow solution to cool too much or it will be difficult to pour gel. Add 10µL Syber Green to the solution and swirl to mix. Pour the melted agarose onto the gel platform until even with platform edge. Allow 20 to 30 minutes for agarose to solidify.

10.6.4 After the gel has solidified, pour enough 1X TBE buffer to completely submerge gel.

10.6.5 Remove the combs without lifting the gel above the liquid level.

10.7 Sample Preparation and Gel Loading:

10.7.1 Using a plastic disposable round bottom microtiter plate, aliquot 3µL BioLine Loading Buffer directly into the bottom of each well for every specimen and control.

10.7.2 For each specimen and control, pipet 10µL of specimen or control into a separate microtiter well, mixing the sample with Loading Buffer.

10.7.3 Load 10µL of the sample mixture into the appropriate well of the gel.

10.7.4 Load 1µL of the BioLine HyperLadder V onto each agarose well following the inoculation of the patient specimens and controls.

10.8 Electrophoresis and Photography:

10.8.1 Electrophorese gel for 1.5 hours at 85 V with a constant current.

10.8.2 Visually verify that the bands have traveled sufficiently over the agarose gel & have not run over the gel. If the bands traveled off the gel, remove gel & repeat the HLA DQα procedure.

10.8.3 Disassemble the gel apparatus and place the gel onto a tray containing enough 1X TBE to completely cover the gel to be photographed.

10.8.4 Clean the UV Transilluminator screen with dH_2O and kimwipes. Place gel on top and focus camera.

10.8.5 Once focused, use appropriate protective eyewear before turning UV Transilluminator on. Position the orange filter over the camera lens.

10.8.6 Slide film into the film holder and expose for 8 to 9 seconds, f4.5 aperture setting. Develop film for 30 seconds before opening.

10.8.7 Alternatively, place Gel in Gel-Doc 2000 and follow manufacturer's instructions to take computer image of gel. Print image to review and document each sample's DNA adequacy for analysis.

10.8.8 Label photograph with tech's initials, or other run identifier, and date. Label each of the controls and patient samples on the photograph.

10.9 HLA DQα Results and Validity:

10.9.1 A distinct white band corresponding to the HLA DQα locus is required for each specimen to be considered adequate for the amplification assay.

10.9.2 A molecular weight marker is included to identify specimen bands due to HLA DQα (MW = 242). The presence of this HLA DQα marker is verifies that each original specimen was adequate for the HIV PCR analysis.

11. TROUBLESHOOTING (HIV DNA PCR and HLA assays):

11.1 Inform the director or the supervisor of any problems with this assay.

11.2 If any negative controls are positive, suspect contamination. Clean up the area and equipment with a solution of 1M NaOH or 1M HCl to remove any contamination with PCR product. Do not release results until the run has been repeated. Repeat the assay to verify results.

11.3 The negative PCR control should have absorbance readings below 0.100. If the A_{450} readings are greater than 0.350, the run should be reviewed with the supervisor and repeated.

11.4 The lab VQA controls +5c (copy standards) are positive/negative controls. The A_{450} readings should be greater than 0.350 but below 2.0; 95% of the time.

11.5 The lab VQA controls +10c and +20c (copy standards) are strong lab posi

tive controls. The A_{450} readings should be positive (preferably >3.0 for the 20 copy and >0.350 for the 10 copy). If the +10c and +20c controls are negative, repeat the assay.

11.6 If the HLA DQα for the patient specimen is negative, the specimen is reported inadequate. There may not be sufficient DNA present or inhibitors of the PCR amplification may interfere with the assay.

12. REFERENCES

12.1 Butcher A, Spadoro J: Using PCR for detection of HIV-1 Infection. Clin Imm Newsletter 12:73:76, 1992

12.2 Cushwa WT, Medrano SF: Effects of Blood Storage Time and Temperature on DNA Yield and Quality. Biotechniques 14:204-207, 1993.

12.3 Roche HIV PCR kit publications, 199**3**.

12.4 Roche information bulletin #3450317001-01

Procedure: ACTG Lab Man Roche Amplicor HIV-1 DNA Test, v1.0

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997