Letter of Amendment # 1

Date: **May 24, 2004**

Re: **LETTER OF AMENDMENT FOR HPTN 034, Version 1.0 October 15, 2001**

HPTN 034 and Substudies 034 A and 034 B

HPTN HIV Incidence and Participant Retention Protocol, Pune, India:

Version 1.0, October 15, 2001

Principle Investigators: **Dr. Robert Bollinger, JHU, USA**

Dr. Sanjay Mehendale, NARI, Pune, India

THE FOLLOWING INFORMATION IMPACTS THE HPTN 034 STUDY AND MUST BE FORWARDED TO YOUR INSTITUTIONAL REVIEW BOARD (IRB)/ETHICS COMMITTEE (EC) AS SOON AS POSSIBLE FOR THEIR INFORMATION AND REVIEW. THIS MUST BE APPROVED BY YOUR IRB/EC BEFORE IMPLEMENTATION.

THE FOLLOWING INFORMATION MAY ALSO IMPACT THE SAMPLE INFORMED CONSENT. YOUR IRB/EC WILL BE RESPONSIBLE FOR DETERMINING THE PROCESS OF INFORMING SUBJECTS OF THE CONTENTS OF THIS LETTER OF AMENDMENT.

PLEASE FILE THIS LETTER AND ANY IRB/EC CORRESPONDENCE IN YOUR REGULATORY FILE AND OTHER PERTINENT FILES. YOU ARE NOT REQUIRED TO SUBMIT THESE DOCUMENTS TO THE PROTOCOL REGISTRATION OFFICE UNLESS THE CHANGES RESULT IN A CHANGE TO THE INFORMED CONSENT FOR YOUR SITE.

Summary of Revisions

Protocol Summary (Page No. 5 of 33):

Population:	Cohort of HIV discordant couples and HIV negative high-risk women will be increased.
Study Duration:	Study duration will be extended by 5.5 months.
Primary Objectives:	Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 1.1	Duration of study will be increased by one year.
Section 1.1	Duration of upcoming studies will be increased by 6 months.
Section 1.2.2	Cohort of HIV discordant couples and HIV negative high-risk women will be increased.
Section 1.3.6	Evaluation of follow up visits will be increased.

Section 2.3 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 3.1 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 3.3.3 Duration of the follow-up period will be increased.

Section 4 (Screening / Enrollment)

Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 4.2 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 4.3 Duration of the follow-up period will be increased.

Section 5.2 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 5.3.1 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 5.3.2 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Section 5.4 Cohort of HIV discordant couples and HIV negative high-risk women will be increased.

Appendix 1.0 Duration of the follow-up period will be increased.

The protocol team roster is updated.

Implementation

Once this Letter of Amendment is approved by the Division of Acquired Immunodeficiency Syndrome (DAIDS) (both the medical officer and Regulatory Affairs Branch), it will be submitted to the DAIDS through the Regulatory Compliance Center (RCC) for informational purposes.

If the HPTN 034 protocol is amended in the future, all changes described in this letter of amendment will be incorporated into the next version.

Modifications are indicated by ~~strikethrough~~ or **bolded** text.

Protocol Summary (Page No. 5 of 33):

Population: ~~400~~ **540** adult HIV uninfected non-sex worker women attending STD clinics and ~~400~~ **530** HIV discordant couples from Pune, India

Study Duration: Accrual will require ~~12~~ **17.5** months and participants will complete ~~one year~~ **one and half years** of follow up

Primary Objectives: To determine the current incidence of HIV among a cohort of ~~400~~ **540** newly identified high-risk women in Pune as well as among ~~400~~ **530** HIV uninfected partners of HIV infected persons.

Section 1.1 Thus the HPTU in Pune is planning for the initiatiation of these 2 clinic-based phase 3 clinical trials over the next ~~two~~ **three** years.

Section 1.1 In anticipation of the initiation of the HPTN 035 trial within the next year and HPTN 052 trial within the next ~~24~~ **30** months.

Section 1.2.2 In addition to the overall HIV sero incidence estimates, this protocol will also provide the first HIV incidence estimate for ~~400~~ **530** HIV uninfected partners of ~~400~~ **530** HIV infected individuals in India.

Section 1.3.6 For this analysis, HIV antibody sero negative patients who received test results within 90 days of screening and were enrolled in the follow-up study were evaluated for a return visit for the time periods 91-180 days**,** ~~and~~ 181 to 360 days**, and 361 to 425 days.**

Section 2.3 This protocol will enroll and follow ~~400~~ **540** women attending STD clinics, who are found at screening to be HIV antibody negative. In addition ~~400~~ **530** HIV uninfected partners of individuals found HIV infected at screening, will be followed together as HIV discordant couples.

As mentioned, we will also attempt to enroll ~~400~~ **530** HIV discordant couples in a prospective cohort, as part of this 034 protocol.

The ~~400~~ **540** HIV-negative women, as well as the ~~400~~ **530** HIV-discordant partners will enroll in the study and then complete quarterly follow-up visits over the course of the ~~12~~ 17.5 months following enrollment.

In addition, if our recruitment goal of ~~400~~ **530** discordant couples has not been met, these seroconverters will be encouraged to refer their sexual partner for HIV screening.

Section 3.1 This study will enroll ~~400~~ **540** adult non-sexworker women attending STD clinics in Pune, India. In addition, ~~400~~ **530** HIV-discordant couples will be enrolled. Thus, a total of ~~800~~ **1070** HIV-uninfected and ~~400~~ **530** HIV-infected participants will be enrolled.

Following initial HIV screening, ~~400~~ **540** HIV-uninfected women and ~~400~~ **530** HIV-discordant couples (total n=~~1200~~ **1600**) will be offered enrollment in this study based upon the following eligibility criteria.

Section 3.3.3 Each partner is available for at least ~~12~~ **17.5** months of study follow-up.

Each couple has stated intention to continue their relationship for the duration of the study [~~12~~ **17.5** months].

Section 4 Of the 1600 HIV-uninfected individuals, we will anticipate enrollment of approximately ~~400~~ **540** new non-sex worker women over the first ~~12~~ **23** months into this follow-up study of HIV serocoversion.

We will also enroll ~~400~~ **530** HIV-discordant couples.

Section 4: (Screening / Enrollment)

(n= ~~1200~~ **1600** enrolled, ~~400~~ **540** HIV –ve women + ~~400~~ **530** HIV discordant couples)

Section 4.2 Of the ~~800~~ **1070** HIV-uninfected participants targeted for enrollment, ~~400~~ **530** will be enrolled with their HIV-infected sexual partners. These ~~400~~ **530** HIV-discordant couples will be consented, enrolled and followed as couples, using identical data forms…as for the ~~400~~ **540** HIV-uninfected women in this study.

Section 4.3 Follow-up visit will occur every 3 months for ~~12~~ **17.5** months.

Section 5.2 As outlined below, we will calculate 6 and 12 month retention rates for the ~~400~~ **540** newly identified and enrolled HIV uninfected women, as well as the ~~400~~ **530** HIV discordant couples

Section 5.3.1 Approximately 2000 new STD patients will be screened over the next year and ~~400~~ **540** newly identified high-risk women and ~~400~~ **530** HIV serodiscordant couples attending the STD clinics in Pune will be enrolled in order to estimate the current HIV incidence.

This 034 protocol will enroll ~~400~~ **540** women, as well as ~~400~~ **530** HIV-uninfected partners of HIV infected persons and follow them for ~~1 year~~ **one and a half years** with an anticipated 90% semiannual retention.

The ~~400~~ **540** high-risk women participants will accrue an estimated ~~348~~ **470** person years of follow-up by ~~July 2003~~ **August 2004**.

Amongst the discordant couples, the ~~400~~ **530** HIV-uninfected partners will accrue ~~348~~ **461** person years of follow-up,

Section 5.3.2 Amongst the ~~400~~ **540** HIV-negative women enrolled in the study, we are targeting a 95% annual retention rate. Using that expected rate, the sample size of ~~400~~ **540** will allow the estimation of the retention rate to within + 2.14% (95%CI). Similarly among the ~~400~~ **530** HIV uninfected members of sero discordant couples we estimate that we will achieve 95% annual retention.

Section 5.4 Overall retention rates will be calculated for all ~~1200~~ **1600** subjects. In addition, the retention rates of the subset of ~~400~~ **540** women and ~~400~~ **530** discordant couples will be separately calculated.

Separate estimates of seroincidence will be calculated for the ~~400~~ **540** women and the ~~400~~ **530** HIV-uninfected partners.

To determine the current incidence of HIV among a cohort of ~~400~~ **540** newly identified high-risk, HIV-negative women in Pune.

In addition, a separate estimate of the HIV incidence among ~~400~~ **530** HIV-uninfected partners of HIV-infected subjects will also be estimated separately.

Appendix 1.0 Follow-up visits will occur every 3 months for ~~12~~ **17.5** months.

Informed Consent Forms: The following addendums have been added to the informed consent forms

Addendum 2 to the Women's Enrollment Consent Form:

Modifications to Study Procedures

There have been 2 modifications to the procedures of this study. This page of the consent form gives you information about these modifications.

A. Objective of the study: You were informed in this paragraph of the consent form that we will be inviting 400 women like you who have a negative HIV/AIDS test to join this study over the next year.

As modification to this section, we will be inviting total 540 women like you who have a negative HIV/AIDS test to join this study.

B. Your part in the study: You were informed that, we are requesting you to come to this clinic every 3 months for the next one year. You have to make a total of 4 more visits.

As per modification, we are requesting you to come to this clinic every 3 months till August 2005. You may have to make more than 4 visits till August 2005.

There have been no other changes to any other information or procedures given to you in this consent form.

Risks: Additional sample size and follow-up period does not put you at any additional risk that has not been informed to you earlier in this consent form.

Signature: This page is intended to make you aware about the changes to the study procedures. Please sign if you have understood this information.

Addendum 2 to the HIV Discordant Couples' Enrollment Consent Form:

Modifications to Study Procedures

There have been 2 modifications to the procedures of this study. This page of the consent form gives you information about these modifications.

A. Objective of the study: You were informed in this paragraph of the consent form that we will be inviting 400 HIV discordant couples like you who have a positive HIV/AIDS test to join this study over the next year.

As modification to this section, we will be inviting total 530 HIV discordant couples like you to join this study.

B. Your part in the study: You were informed that we are requesting you to come to this clinic every 3 months for the next one year. You have to make a total of 4 more visits.

As per modification, we are requesting you to come to this clinic every 3 months till August 2005. You may have to make more than 4 visits till August 2005.

There have been no other changes to any other information or procedures given to you in this consent form.

Risks: Additional sample size and follow-up period does not put you at any additional risk that has not been informed to you earlier in this consent form.

Signature: This page is intended to make you aware about the changes to the study procedures. Please sign if you have understood this information.

Clarification Memo #1

27 December 2002

Summary of Revisions

1. Screened volunteers will be called for HIV test results and post-test counseling 7-14 days after their screening visit. The volunteers having HIV negative serology within 22 days will be enrolled.

2. Follow-up visits will occur every 3 months for one year. The participants coming within 22 days of their scheduled follow-up visit will be taken for follow-up.

Revision to Clarification Memo #1

8 May 2003

Summary of Revisions

1. Screened volunteers will be called for HIV test results and post-test counseling 7-14 days after their screening visit. The volunteers having HIV negative serology with 22 days will be enrolled.

2. Follow-up visits will occur every 3 months for one year. The participant coming within 22 days of their scheduled follow-up visit will be taken for follow-up. After 22 days it will be considered as a missed visit.

Clarification Memo #2

7 March 2003

Summary of Revisions

Sera of HPTN 034 study participants will be initially screened with ELISA kits either commercially available or supplied by National AIDS Control Organization (NACO), India. Sera that are positive will be confirmed by re-testing with commercially available or NACO supplied kits.

Clarification Memo #3

20 October 2003

Summary of Revisions

The blood samples taken from HPTN 034A laboratory sub-study participants (acute serconverters) will be tested for complete blood count (CBC) and CD4 cell count at enrollment and at all study visits for the first year of follow-up, and once every six months for the second year of follow-up.

Clarification Memo # 4

20 October 2003

Summary of Revisions

To update the contact numbers provided in the PROBLEMS OR QUESTIONS Section of the HPTN 034 Informed Consents (ICF), Version 1.0, October 15, 2001, to reflect the recently modified telephone dialing system requiring the addition of the number "2" in front of the original 7-digit phone number.

HIV Prevention Trials Network

CLARIFICATION # 1, REVISION #1

HPTN 034, Version 1.0

HIV Prevention Preparedness Study

Original date: 27 December 2002
Revision#1 date: 8 May 2003

Summary of Revisions

1. Screened volunteers will be called for HIV test results and post-test counseling 7-14 days after their screening visit. The volunteers having HIV negative serology within 22 days will be enrolled.

2. Follow up visit will occur every 3 months for one year. The participants coming within 22 days of their scheduled follow up visit will be taken for follow up. **After 22 days it will be considered as a missed visit.**

Rationale

The current protocol specifies the time frame of 7-14 days for HIV test result visit. However it does not specify the time period after which the screened participant become ineligible and should be rescreened.

The protocol also does not specify variation of time period that can occur for the scheduled follow up visit.

Implementation

The following modifications are made to Protocol HPTN 034 (version 1.0, dated October 15,2001) to implement the revisions described above. These modifications will be incorporated into the body of the protocol in the next version.

1. Section 2.3 Study design, page 13, paragraph 1

The passage 'scheduled to receive their HIV tests and post test counseling at a study enrollment visit 7-14 days later.' will be changed to 'scheduled to receive their HIV tests and post test counseling at return for test result visit 7-14 days later.' The additional sentence which is to be added as the last line of the same paragraph reads as 'Regardless of the number of screening visits required enrollment of HIV negative participant must be completed within 22 days from the time of first screening tests and exams.'

Section 4.2 Enrollment, page 19, paragraph 2

Dr. D. S. SHROTRI
Chairman
Ethical Committee
National AIDS Research Institute

12.6.03

The passage 'who will answer all questions posed by patients' will be changed to 'who will answer all questions posed by patients provided the HIV seronegative participants return to the study clinic within 22 days of their screening visit.'

2. Section 4.3 Follow-up visits, page 19, paragraph 3

The following sentence will be added after the passage 'The follow up visit will occur every three months for one year' which reads as 'The follow up visit will occur every three months for one year. The participants coming within 22 days of their scheduled follow up visit will be taken for follow. After 22 days it will be considered as missed visit.'

Approved
15 Oct 03
12-8-03

Dr. D. S. SHROTRI
Chairman
Ethical Committee
National AIDS Research Institute

HIV Prevention Trial Network

CLARIFICATION MEMO # 2

HPTN 034, VERSION 1.0:

HIV Prevention Preparedness Study

March 7, 2003

Summary of revision

Sera of HPTN 034 study participants will be initially screened with ELISA kits either commercially available or supplied by National AIDS Control Organization (NACO), India. Sera that are positive will be confirmed by re-testing with commercially available or NACO supplied kits.

Rationale

The HPTN 034 study was initiated at Pune site on 16th September 2002. The current protocol specifies that certain brands of HIV ELISA kits be used for initial testing and confirmation. However, it is practically difficult to use these specific kits due to inconsistent availability in the market. NARI has been using commercially available or NACO-supplied kits for the last 9 years with satisfactory results. The NARI CORE laboratory has certified all HIV ELISA test kits via an internal quality control procedure, whether commercially available or NACO-provided. Hence, we would like to propose a change in the protocol text allowing for any brand of ELISA kit to be used for the study.

Implementation

The following modification will be made to Protocol HPTN 034 (Version 1.0 dated October 15, 2001) to implement the revisions described above. This modification will be incorporated into the body of the protocol in the next version.

Section 4. Study Procedures, 4.4 Detection of HIV infection, paragraph 1, Lines 2-5 (page 20 of 33)

Original Text:

"Sera will be initially screened with the Genetic Systems combination ELISA kit for detection of HIV-1 and HIV-2, which is our current ELISA screening method. Specimens that are positive by Genetic Systems ELISA will be confirmed for HIV-1 or HIV-2 infection by re-testing with a different ELISA (UBI HIV 1/2 EIA, United Biomedical, USA)."

New Text:

"Sera of HPTN 034 study participants will be initially screened with ELISA kits either commercially available or supplied by National AIDS Control Organization (NACO), India. Sera that are positive will be confirmed by re-testing with commercially available or NACO supplied kits."

HIV Prevention Trials Network

CLARIFICATION # 1

HPTN 034, version 1.0:

HIV Prevention Preparedness Study

December 27, 2002

Summary of Revisions

1. **Screened volunteers will be called for HIV test results and post-test counseling 7-14 days after their screening visit. The volunteers having HIV negative serology within 22 days will be enrolled.**

2. **Follow up visit will occur every 3 months for one year. The participants coming within 22 days of their scheduled follow up visit will be taken for follow up.**

Rationale

The current protocol specifies the time frame of 7-14 days for HIV test result visit. However it does not specify the time period after which the screened participant become ineligible and should be rescreened.
The protocol also does not specify variation of time period that can occur for the scheduled follow up visit.

Implementation

The following modifications are made to Protocol HPTN 034 (version 1.0, dated October 15,2001) to implement the revisions described above. These modifications will be incorporated into the body of the protocol in the next version.

1. **Section 2.3 Study design, page 13, paragraph 1**

The passage ' scheduled to receive their HIV tests and post test counseling at a study enrollment visit 7-14 days later.' Will be changed to 'scheduled to receive their HIV tests and post test counseling at return for test result visit 7-14 days later.' The additional sentence which is to be added as the last line of the same paragraph reads as ' Regardless of the number of screening visits required

enrollment of HIV negative participant must be completed within 22 days from the time of first screening tests and exams.'

2. **Section 4.2 Enrollment, page 19, paragraph 2**

The passage 'who will answer all questions posed by patients' will be changed to 'who will answer all questions posed by patients provided the HIV seronegative participants return to the study clinic within 22 days of their screening visit.

3. **Section 4.3 Follow-up visits, page 19, paragraph 3**

3. **The following sentence will be added after the passage 'The follow up visit will occur every three months for one year' which reads as 'The follow up visit will occur every three months for one year. The participants coming within 22 days of their scheduled follow up visit will be taken for follow up otherwise they will be rescreened.**

HPTN HIV Incidence and Participant Retention Protocol
Pune, India

HPTN Protocol 034
A Study of the HIV Prevention Trials Network

Sponsored by:

Division of AIDS
US National Institute of Allergy and Infectious Diseases
US National Institutes of Health

Protocol Chair(s):

Robert C. Bollinger MD, MPH
Sanjay M. Mehendale, MD. MPH

Final Version 1.0
15 October 2001

HPTN Protocol 034
HPTN HIV Incidence and Participant Retention Protocol
Pune, India

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
CAB	Community Advisory Board
DAIDS	Division of AIDS
HIV	Human Immunodeficiency Virus
HPTN	HIV Prevention Trials Network
HPTU	HIV Prevention Trials Unit
IRB	Institutional Review Board
JHU	Johns Hopkins University, USA
NARI	National AIDS Research Institute, Pune, India
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
PPS	Prevention Preparedness Study
SDMC	Statistical and Data Management Center
STD	Sexually Transmitted Disease
US	United States

HPTN Protocol 034
HPTN HIV Incidence and Participant Retention Protocol
Pune, India

PROTOCOL TEAM ROSTER

National AIDS Research Institute Pune, India

Mehendale, Sanjay M	*M.D., M.B.B.S., M.P.H.*	*Principal Investigator*
Paranjape, Ramesh S	*Ph.D.*	*Co-Investigator*
Risbud, Arun R.	*M.D., M.B.B.S., M.P.H.*	*Co-Investigator*
Tripathy, Srikanth	*M.D., M.B.B.S*	*Co-Investigator*
Gangakhedkar, Raman R	*M.B.B.S., D.C.H., M.P.H.*	*Co-Investigator*
Divekar, A.D.	*M.B.B.S., D.M.V.*	*Co-Investigator*
Sahay, Seema	*Ph.D.*	*Co-Investigator*
Ghate, M.V.	*M.B.B.S., D.C.H.*	*Co-Investigator*
Kulkarni, S.S.	*Ph.D.*	*Co-Investigator*
Joshi S. N.	*M.B.B.S.*	*Co-Investigator*
Brahme R. G.	*B. Sc., M. C. M.*	*Co-Investigator*
Joglekar N.	*M. Sc.*	*Co-Investigator*
Kohli R.	*Ph. D.*	*Co-Investigator*
Thakar M. R.	*Ph. D.*	*Co-Investigator*
Yadav, R.	*M. Sc. M. Phil*	*Co-Investigator*

Johns Hopkins University, Baltimore, USA

Bollinger, Robert C.	*M.D., M.P.H.*	*Principal Investigator*
Brookmeyer, Ronald	*Ph.D.*	*Co-Investigator/Biostatistician*
Quinn, Thomas	*M.D., M.Sc.*	*Co-Investigator*
Shepherd, Mary	*M.S.*	*Co-Investigator*
Celentano, David	*Ph.D.*	*Co-Investigator*
Reynolds, Steven	*M.D.*	*Co-Investigator*
Propper, Lidia	*M.S.*	*Data Manager*
Gupte, Nikhil	*M.S.*	*Data Analyst*
TBA		*Protocol Coordinator*

HPTN Protocol 034
HPTN HIV Incidence and Participant Retention Protocol
Pune, India

A Study of the HIV Prevention Trials Network

Sponsored by:

Division of AIDS (DAIDS)
US National Institute of Allergy and Infectious Diseases (NIAID)
US National Institutes of Health (NIH)

I, the Principal Investigator, agree to conduct this study in full accordance with the provisions of this protocol. I agree to maintain all study documentation for a minimum of five years from the end of the study, unless directed otherwise by the HPTN CORE. Publication of the results of this study will be governed by HPTN and DAIDS policies. Any presentation, abstract, or manuscript will be made available by the investigators to the HPTN Manuscript Review Committee and DAIDS for review prior to submission.

I have read and understand the information in this protocol and will ensure that all associates, colleagues, and employees assisting in the conduct of the study are informed about the obligations incurred by their contribution to the study.

_____

Name of Principal Investigator

_____ _____

Signature of Principal Investigator **Date**

HPTN Protocol 034

HPTN HIV Incidence and Participant Retention Protocol, Pune, India

PROTOCOL SUMMARY

Design: This protocol is a cohort study of HIV incidence in patients attending STD clinics in Pune, who will be eligible for screening and participation in 2 upcoming HPTN clinical trials of vaginal microbicides (HPTN 035) and antiretroviral drugs (HPTN 052).

Population: 400 adult HIV-uninfected non-sex, worker women attending STD clinics and 400 HIV discordant sexual couples from Pune, India.

Study Duration: Accrual will require 12 months and participants will complete one year of follow-up. Therefore, the entire study should be completed within approximately 24 months.

Primary Objectives:

1. To determine the current incidence of HIV among a cohort of 400 newly identified high-risk, women in Pune, as well as among 400 HIV-uninfected partners of HIV-infected persons.

2. To determine the retention rate in high-risk women, as well as HIV discordant couples, among a target population eligible for screening for upcoming HPTN 035 and 052 trials.

Primary Endpoints:

- Current HIV seroconversion rates in HIV-uninfected participants.

- Retention rates in high-risk women, as well as discordant couples.

Secondary Objectives:

1. To assess risk factors for HIV and other STDs.

2. To identify and address potential barriers to participation and high- level retention (>95% 1 year retention) for the upcoming HPTN clinical trials.

Secondary Endpoints:

- High-risk behaviors for HIV and STDs in newly enrolled participants and change in high-risk behavior over time.

- Design of effective strategies to retain participants and overcome barriers to participation for upcoming HPTN clinical trials.

1 BACKGROUND AND RATIONALE

1.1 Timelines for upcoming HPTN clinical trials in Pune, India

The United States (US) National Institutes of Health (NIH) established the HIV Prevention Trial Network to develop and test non-vaccine strategies to prevent the spread of HIV. The HPTN is the largest multicenter research network dedicated to HIV prevention. There are 26 research sites and sub-sites located in Africa, Asia, Europe, South America, and the US, including Pune, India.

In response to the specific recommendations for the HPTU in Pune, by the HPTN Executive Committee, this protocol will undertake specific studies required for the successful initiation and completion of the following two upcoming HPTN clinical trials:

A) Phase III multi-national microbicide trial (HPTN 035)

B) Multinational randomized study of antiretroviral therapy for prevention of HIV transmission in HIV-discordant couples (HPTN 052)

Thus, the HPTU in Pune is planning for the initiation of these 2 clinic-based Phase III clinical trials over the next 2 years. These proposed clinic-based HPTN trials will require additional site-specific research, as outlined in this protocol 034. In light of the current timelines proposed for approval, implementation and completion of these HPTN clinical trial protocols, we anticipate that we will be able to accomplish each of the two primary objectives proposed for this study prior to the initiation of the clinical trials (See 4.9). In the event that any of these clinical trials are ready for initiation prior to completion of these proposed activities, we will conduct an interim analysis and provide this to the HPTN PRC and EC. Based upon their recommendations, we will either continue our proposed activities or will terminate those activities to facilitate rapid initiation of the HPTN clinical trials in Pune. Although the details and timelines for these 2 clinical trials are pending final protocol development and reviews, we anticipate that the preparatory activities proposed in this 034 protocol will facilitate the efficient transition to each of the clinical trials and that the initiation of the trials will be well coordinated. In addition, the activities proposed in this 034 protocol are designed to facilitate the final development of the 2 clinical trial protocols, to specifically prepare the Pune site for each of these trials and to prepare the community for the trials, as well.

Previous prevention trial planning efforts have indicated that Phase III studies of HIV prevention interventions will require the participation of large numbers — from several hundred to several thousand — of persons at high risk for HIV infection. In addition to accruing large numbers of participants, research centers conducting Phase III HIV prevention studies must retain participants in extended periods of follow-up — from several months to several years — in order to preserve the statistical power of the study as well as avoid potentially biased results that may not accurately reflect the impact of the intervention in the target population. Some have recently argued that a greater than 95%

1 year retention is required for any HPTN Phase III clinical trial. Our HPTU in Pune is committed to achieving the enrollment and retention required for such a trial.

The design of Phase III HIV prevention trials to be conducted in the HPTN will depend on the efficacy or effectiveness of intervention being studied as well as the interplay of the four parameters referenced above: the number of participants enrolled, the HIV incidence rate among enrollees, the duration of follow-up, and the number of participants retained in follow-up.

Our site in Pune has already conducted two Phase I microbicide trials, and is well prepared for initiating the upcoming PRO 2000/5 Gel (P) Phase I trial (HPTN 047). We are currently preparing the protocol, in anticipation of initiating this trial within the next 4-6 months. In anticipation of the initiation of the HPTN 035 trial within the next year and the HPTN 052 trial within the next 24 months (see time-line chart in Section 4.9 below), we have identified a number of specific research objectives that we believe are necessary to ensure the successful and efficient initiation of these HPTN trials. This study will also provide new data important for the design of future HIV prevention trials.

1.2 Key Research Questions for Successful Initiation of HTPN Trials in Pune

The rationale for this protocol is based upon our identification of a number of key research questions that will directly impact the design and implementation of HPTN prevention trials in Pune and goal of this preparedness protocol is to try and answer these questions.

1.2.1 What is the current HIV incidence of high-risk, non-sexworker women attending STD clinics and in Pune, India?

In order to successfully undertake the upcoming HPTN 035 trial, we require a current estimate of HIV incidence in the high-risk women representative of those potentially eligible for enrollment in this trial. As outlined below, we have successfully identified and enrolled more than 2500 high-risk men and women in multiple cohort studies in Pune, since 1993. These cohort studies were supported in the past by the PAVE and HIVNET projects. A time-trend analysis has suggested that the HIV incidence may be decreasing in recently enrolled women attending the STD clinics in Pune. However, our recent cohort activities have been limited as a result of the discontinuation of the HIVNET study. Therefore, a current measurement of HIV incidence and the risk factors associated with HIV infection will be necessary for sample size estimates and assessment of the current capacity for the HPTU in Pune to identify and enroll eligible participants for this upcoming HPTN trial. This protocol will provide a current estimate of the HIV seroincidence of high-risk women representative of those who may participate in the HPTN 035 trial.

1.2.2. What is the current HIV incidence rate in the HIV-uninfected partners of HIV-infected STD patients in Pune?

In addition to the overall HIV seroincidence estimates, this protocol will also provide the first HIV incidence estimate for 400 HIV-uninfected partners of 400 HIV-infected individuals in India. The current proposed study design of HPTN 052 will require the identification and enrollment of HIV discordant couples. Our site has already participated in a small HIV discordant couples study supported by the HIVNET. However, we have not assessed our capacity to enroll and retain the large number of discordant couples that will be required for this Phase III clinical trial. In addition, we have not measured the HIV transmission rate in the exposed partners of HIV-infected individuals in Pune. Timely assessment of the HIV incidence rate in these exposed partners will be required for estimation of sample size for the upcoming trial.

1.2.3. What is the current retention rate of newly enrolled high-risk women and discordant couples?

Also, as outlined later, we have recently achieved a high retention rate in a number of small prospective studies and Phase I clinical trials in Pune. In addition, our retention rate in the large observational cohort has recently greatly increased. However, we anticipate that a successful HPTN clinical trial will require large numbers of participants with a retention rate of at least 90-95% at one year. Thus, we require measurement of the retention rate of newly enrolled high-risk women, as well as an opportunity to identify procedures to ensure very high follow-up of newly enrolled participants. In addition, we require an assessment of our ability to enroll and retain HIV-discordant couples for the proposed HPTN 052 trial.

1.3 Current Capacity for Screening, Enrollment and Follow-up for Multiple HPTN Clinical Trials in Pune

Undertaking multiple Phase I/II and III HPTN clinical trials will require sites with the capacity for screening, enrollment and intensive follow-up of both high and low-risk participants, as well as the clinical and laboratory capacity to assess toxicity and acceptability of potential interventions. Our capacity for such trials in Pune is demonstrated by our completion of three clinical trials and two prospective follow-up studies of patients with recent HIV infection. Enrollment and retention rates for these studies are summarized in the table below.

Screening Enrollment and Follow-up: NARI/JHU Clinical Trials/ Prospective Studies

Study	Design	Subjects	Status Date	Screened	Eligible	Enrolled	Follow-up Duration	Retention
HIVNET 009	Phase I Clinical Trial Multinational	Low risk women	Complete 1998	39	30	21	1 month	20 (95%)
N-9 Microbicide	Phase I Clinical Trial Single Site	Low risk women	Complete 1998	37	26	23	1 month	23 (100%)
HIVNET 013	Phase I Clinical Trial Multinational	Discordant couples	Complete 1999	45	33	14	3 months	14 (100%)
CTL Activity	Cohort Study	Recent HIV Infection	Complete 1998	NA	16	12	3 months	12 (100%)
Acute HIV Infection	Cohort Study	Acute HIV Infection	Ongoing 1999	2074	40	35	24 months	33 (94%)

1.3.1. HIVNET 009: Phase I Clinical Trial of Buffergel [Reprotect] Vaginal Gel. The objective of this study was to assess the toxicity and acceptability of Buffergel Vaginal Gel on genital mucosa in low-risk HIV-negative women.

1.3.2. Phase I Study of Today N-9 Vaginal Microbicide. The objective of this study was to assess the toxicity and acceptability of a commercially available Indian made N-9 microbicide ("TODAY"). Study procedures were similar to the HIVNET 009 study. This was a single site study in Pune and the first Phase I clinical trial of any microbicide undertaken in India.

1.3.3. HIVNET 013: Phase I Trial to Increase Condom Use by HIV Discordant Couples. This recently completed multi-national HIVNET-sponsored clinical trial in HIV discordant couples was undertaken in India, Thailand and Uganda.

1.3.4. CTL Activity in HIV-infected Individuals in India. (1R03TW00898-01). The objective of this study was to measure and characterize the HIV-specific immune response in individuals with recent HIV infection.

1.3.5. Pathogenesis of Acute, Primary HIV Infection (R01 AI41369-01-A1). In light of the successful accrual for the CTL study above, an intensive pathogenesis study of the virologic and immunologic aspects of acute HIV infection was initiated. Individuals with recent HIV infection are identified through their participation in our cohort study of high-risk patients attending three STD clinics, mentioned above. New STD patients and high-risk participants in our cohort study are now routinely immediately screened for p24 antigen, if their HIV antibody test is negative. Thirty-five subjects with acute HIV infection have been enrolled in this prospective follow-up study. These individuals agree to have multiple phlebotomies and clinical examinations frequently for the first 3 months following acute HIV infection and are followed for 24 months.

1.3.6. Screening Enrollment and Follow-up: NARI/JHU Clinical Trials/ Prospective Studies

We feel it is important to obtain a very current and accurate estimate of HIV incidence in the population in Pune proposed for the three upcoming clinic-based HPTN trials in Pune. Presented in the table below are the HIV incidence rates observed by year of enrollment for participants enrolled in our cohort study of HIV-uninfected patients presenting to the STD clinics in Pune. These prior cohort studies were supported by the NIH Preparation for HIV Vaccine Evaluation (PAVE) and HIVNET, as well as an R01 grant to investigate the immunological and virological characteristics of early HIV infection.

HIV Incidence Within 1 Year of Follow-up, by Year of Enrollment

Year Enrolled	Person-Years	HIV incidence rate	(95% CI)
1993	406.05	10.84	(7.87-14.56)
1994	408.30	9.06	(6.38-12.49)
1995	345.67	8.97	(6.09-12.75)
1996	329.85	8.79	(5.89-12.66)
1997	140.01	10.00	(5.46-16.80)
1998	142.38	5.62	(2.42-11.07)
1999	173.01	1.16	(0.14-4.17)

On average, approximately 100 new high-risk individuals have been enrolled in our cohort, every quarter for the past 2 years. Our recent recruitment of new high-risk individuals also reflects a reduction in support and effort for the recruitment of a large cohort, in the absence of a clinical trial in Pune.

Earlier in our studies in Pune, the follow-up rates in this observational cohort were not optimal for clinical trials. Our behavioral studies suggested that the perceived benefit for participation in an observational study were not as clear or compelling to the high-risk patients in our community, as they were for participation in a specific clinical trial. This was substantiated by our subsequent observation that the follow-up rates for our cohort studies clearly did not predict the excellent follow-up rates we have demonstrated in our subsequent clinical trials. It is very clear from our previous success with enrollment and retention of high risk cohorts outlined in the previous section, that when specific clinical trial interventions are proposed and understood by our community, our cohorts are willing to enroll in clinical trials and follow-up rates are excellent.

In addition, over the past 2 years, we have made modifications in our clinics specifically designed to improve the follow-up rates in our observational cohort of high-risk HIV infected volunteers. These procedural changes focused primarily on improving the informed consent process for this observational study. Prior to April 1998, consent for HIV screening and enrollment in our cohort studies were both obtained at the screening visit. Since that time, consent for HIV testing only is obtained at the screening visit. A separate consent for enrollment in our observational study is obtained when HIV-uninfected individuals return within 2 weeks for their post-test counseling. In addition to

a statistically significant increase in the percentage of patients who return for their HIV test results after initial screening, there has been a dramatic improvement in our follow-up rates for recent enrollees in our observational cohort. Since the protocol was modified recently, we only have sufficient data to compare the 6-month follow-up/retention rates for HIV-uninfected volunteers enrolled prior to April 98 with those enrolled after April 98. For this analysis, HIV antibody seronegative patients who received test results within 90 days of screening and were enrolled in the follow-up study were evaluated for a return visit for the time periods 91-180 days and 181 to 360 days. Overall, the 6-month retention rate for the new high risk HIV-uninfected subjects enrolled in our observational cohort study since April 98 is 81%, compared with 29% for HIV-uninfected volunteers enrolled prior to April 1998. Our analysis of exit interviews of study participants suggests that the primary reason for the improved follow-up was that more recent participants more clearly understood the expectations for participation in a research study. Separating and enhancing the HIV screening and enrollment consent processes appears to be the primary reason for this improvement. Encouraged with the success of this, we are also planning to focus on designing a pictorial consent or in the form of a short video clip that could be easily understood by less educated people.

Our previous experience with enrollment and follow-up for clinical trials suggests that this 81% retention rate will be increased and sustained with effective community education about the specific risks, benefits and objectives of participating in HPTN clinical trials. However, this HPTN protocol will target a goal of at least 90-95% 1 year retention in our observational cohort for newly identified and enrolled high-risk individuals, in anticipation of the need for this level of retention for the upcoming HPTN trials. With a current follow-up rate of >80% for our observational study of HIV transmission and follow-up rates of >90% for our other prospective clinical studies, including three HIV clinical trials, we are confident that this protocol will help us identify the procedures required to achieve the excellent retention of participants required for the HPTN 035 and 052 trials.

One of the proposed HPTN trials for Pune (HPTN 052) will enroll HIV discordant couples. The Pune site has some experience through the HIVNET 023 couples study with screening, enrollment and follow up of discordant couples. However, this 034 protocol specifically includes efforts to identify and enroll up to 400 HIV discordant couples in an observational cohort study. This will provide essential experience and a preliminary base for the enrollment of discordant couples into future HPTN clinical trials. NARI has a HIV Reference Clinic facility on the premises of National Institute of Virology, Pune. This site is proposed for recruiting HIV discordant couples in various clinical trials. In addition, we have also successfully negotiated a clinic space in Talera Hospital in Chinchwad, an adjoining Municipal hospital and we have initiated an HIV reference clinic in this center that will also serve as a referral source for HIV discordant couples. In addition, through community outreach to local physicians, hospitals and clinics, NARI has become a regional reference center for the care of HIV infected individuals. We anticipate that this referral base will also provide additional opportunities to identify HIV-infected index partners for enrollment of discordant couples.

Finally, we have a long-standing clinical infrastructure and established procedures for identifying and enrolling high-risk men and women, who will be eligible for screening for the upcoming HPTN trials in Pune. It has also been explained above that we also have plans to expand the clinical facility. In light of the anticipated initiation of at least 2 of these trials within the next year, we feel it will be most efficient and cost-effective to maintain our current procedures for screening and enrollment of new STD patients in anticipation of the timely initiation of the HPTN trials. We are hoping to have a continued support to our current clinic-based activities, because this is what our community and the Community Advisory Board will expect and this also will allow us to hire and retrain the clinic staff within a few months for the upcoming HPTN trials. Taken together with the need for current estimates of HIV incidence in the cohort and community populations and the need for data to ensure high retention rates, we feel that this protocol is important for the HPTN generally and for our HPTU in Pune, in particular.

2 STUDY OBJECTIVES AND DESIGN

2.1 Primary Objectives

The overall goal is to ensure that the HPTU in Pune is optimized for the initiation, enrollment and retention of participants for two up-coming HPTN clinical trials. The specific primary objectives are:

- To determine the current incidence of HIV among a cohort of newly identified high-risk, HIV-negative non-sexworker women, as well as among the HIV-uninfected partners of HIV-infected persons.

- To determine the retention rate in high-risk women, as well as HIV discordant couples.

2.2 Secondary Objectives

- To determine risk factors for HIV transmission in high-risk women and among HIV discordant couples.

- To identify and address potential barriers to participation and high- level retention (>95% 1 year retention) for the upcoming HPTN clinical trials.

2.3 Study Design

This protocol is a cohort study of HIV incidence in persons attending STD clinics in Pune, who will be eligible for screening for 2 upcoming HPTN clinical trials of vaginal microbicides (HIV-uninfected female spouses of male STD patients or HIV uninfected female STD patients) and antiretroviral drugs (HIV discordant couples).

After providing written informed consent, potential study participants will undergo eligibility screening, including administration of a questionnaire, physical examination and HIV antibody testing. Presumptively-eligible participants (based on the screening questionnaire) will complete an assessment of their HIV risk behaviors, undergo phlebotomy for HIV antibody testing at the local laboratory, and be scheduled to receive their HIV test results and post-test counseling at a study enrollment visit 7-14 days later. At their post-test visit, participants will be assessed for their eligibility for enrollment in the cohort of HIV-uninfected non-sex worker women or the cohort of discordant couples.

This 034 protocol will enroll and follow 400 women attending STD clinics, who are found at screening to be HIV antibody negative. These women will not be eligible for enrollment in the cohort, if they have reported a history of commercial sex work. In addition, 400 HIV-uninfected partners of individuals found to be HIV-infected at screening, will be followed together as HIV discordant couples. When screened patients return for their HIV test results, HIV-uninfected women will be invited to participate in this cohort study. Men or women who test HIV-positive at screening will be counseled, referred to available medical and psychosocial services, and invited to refer their primary sexual partner (usually spouse) to the clinic for HIV testing. As mentioned, we will also attempt to enroll 400 HIV discordant couples in a prospective cohort, as part of this 034 protocol. The 400 HIV-negative women, as well as the 400 HIV-discordant partners will enroll in the study and then complete quarterly follow-up visits over the course of the 12 months following enrollment. At these follow-up visits, in addition to physical examinations, participants' HIV risk behaviors (since the last visit) and HIV serostatus will be ascertained (Note: the HIV-infected partners in the discordant couples sub-group will not have repeat HIV screening). HIV testing also may be performed between scheduled follow-up visits, at participant request, throughout the course of the study.

Participants will receive their follow-up HIV test results and post-test counseling at post-test visits 7-14 days after their pre-test visits. For pre and post test counseling, we will utilize the expanded HIV pre- and post-test counseling that we developed for HIVNET 013 for the discordant couples study in Pune. The counseling consists of individual HIV pre-test counseling based on the WHO/CDC guidelines that have been adapted to India. In addition, we stress confidentiality issues in depth as well as disclosure within couples of HIV serostatus. Risk reduction counseling is the focus, and condoms are provided to couples at no charge at all visits. This counseling package was based on the Center for AIDS Prevention Studies (UCSF) module prepared for the 3-country VCT study for developing countries.

Participants who HIV seroconvert during the course of the study, will be counseled, discontinued from this study, referred to available medical and psychosocial services, and referred to other available research studies, including the HPTN 034A substudy (See appendix). In addition, if our recruitment goal of 400 discordant couples has not been met, these seroconverters will be encouraged to refer their sexual partners for HIV screening. If their partners are HIV negative, seroconverters will also be eligible to continue follow-up as part of the HIV discordant couples sub group. HIV pre-test, risk reduction, and post-test counseling will be provided at each testing time-point.

3 STUDY POPULATIONS

3.1 Recruitment Procedures

This study will enroll 400 adult non-sexworker women attending STD clinics in Pune, India. In addition, 400 HIV-discordant couples will be enrolled. Thus, a total of 800 HIV-uninfected and 400 HIV-infected participants will be enrolled.

Patients attending three established outpatient STD clinics in Pune, India will be recruited into this prospective cohort study. In addition, NARI has a HIV Reference Clinic facility on the premises of National Institute of Virology, Pune. This site will also provide a referral base for recruiting HIV discordant couples. In addition, we have also successfully negotiated a clinic space in Talera Hospital in Chinchwad, an adjoining Municipal hospital and we are hoping to initiate a HIV reference clinic to this center that could screen and enroll high-risk women and HIV discordant couples.

There are two basic risk groups to be recruited into this cohort study, reflecting our goal to specifically prepare for initiation of the HPTN 035 and 052 trials. The two groups in this cohort study will be

A) High-risk women, who are not commercial sex workers. These include women presenting to the STD clinics and referred women who are the spouses of the male STD patients. (Male STD patients are encouraged to refer their partners/wives to the clinic for evaluation and treatment of STDs and HIV screening).

B) HIV discordant couples. These will be expected to be primarily HIV-infected men identified at screening and their HIV-uninfected wives. We do not expect to identify very many HIV-discordant couples where the woman is the HIV-infected index case and the man is HIV-uninfected. However, such discordant couples would also be eligible for enrollment. As outlined below (Section 3.3), discordant couples will be enrolled following a three-step procedure (index partner, exposed partner and discordant couples) that involves inclusion criteria and informed consent at each step. Also, it is understood that index partners may have more than one sexual partner. This protocol only requires participation of one such regular partner. The index partner will be counseled to disclose their HIV status to all partners, but only one of those partners will be enrolled in the study.

Following initial HIV screening, 400 HIV-uninfected women and 400 HIV-discordant couples (total n=1200) will be offered enrollment in this study based upon the following eligibility criteria.

3.2 Inclusion Criteria HIV-uninfected Women (n=400)

HIV-uninfected individuals attending the STD clinics (n=400) must meet all of the following criteria in order to be eligible for inclusion in this cohort study:

- Age 18 and older
- Able and willing to provide written informed consent for HIV testing and to take part in the study.
- HIV seronegative by licensed ELISA.*
- Available for at least 12 months of study follow-up.
- Must agree to receive their HIV test results.

*Note: Individuals with only a single ELISA test positive, i.e. with a negative confirmatory ELISA will be considered HIV seronegative and eligible for enrollment.

3.3 Inclusion Criteria HIV-discordant Couples (n=400 couples)

3.3.1. HIV-infected partners (index partner) of HIV-uninfected patients attending the STD clinics must meet all of the following criteria in order to be eligible for inclusion in the cohort study:

- Age 18 and older
- Able and willing to provide written informed consent for HIV testing and to take part in the study.
- HIV seropositive by 2 different licensed ELISAs
- Willing to receive their HIV test results.
- Available for at least 12 months of study follow-up
- Presently in a sexual relationship with the same partner for at least three months duration
- Intend to remain with this partner for the duration of the study
- Willing to identify his/her sexual partner and disclose his/her HIV status to partner.
- Willing to attempt to recruit their partner

3.3.2. Partners of HIV-infected index partners who meet eligibility criteria 3.3.1. above must meet all of the following criteria in order to be eligible for HIV screening:

- Age 18 and older
- Able and willing to provide written informed consent for HIV testing
- HIV seronegative by licensed ELISA
- Willing to receive their HIV test results.

3.3.3. HIV-discordant couples who meet initial criteria 3.3.1 and 3.3.2 above must also meet all of the following criteria in order to be eligible for inclusion in the cohort study:

- Each partner agrees to participate in the study together
- Each partner is able and willing to provide written informed consent to take part in the study.
- The partners have been in a sexual relationship for at least the prior 3 months.
- Each partner is available for at least 12 months of study follow-up
- Each couple has stated intention to continue their relationship for the duration of the study (12 months).
- Each partner is willing and able to attend each scheduled follow-up study visit

3.4 Exclusion Criteria

Persons who meet any of the following criteria will be excluded from the studies:

- HIV-uninfected women who report a history of prior commercial sex work will not be eligible for enrollment either as part of the female cohort or as an HIV-uninfected partner of an HIV-infected man. However, HIV-infected female commercial sex workers can be enrolled in the HIV-discordant partners study with their HIV-uninfected male primary sexual partner

- Have an obvious psychological/psychiatric disorder that would preclude provision of informed consent or otherwise contraindicate study participation.

- Have any other condition that, in the opinion of the investigator, would preclude provision of informed consent, make participation in the study unsafe, complicate interpretation of study outcome data, or otherwise interfere with achieving the study objectives.

- Discordant couples who report a history of domestic violence will be excluded from the study.*

*Because the women's interview will raise issues of physical, sexual, or psychological abuse, the interviewers will be trained to discuss these emotionally charged issues, and will be able to refer women for help. Agencies in Pune will be identified as referral resources. A psychologist with experience counseling victims of domestic violence will be hired as a training consultant. Maintaining the privacy of counseling interviews and research interviews is essential to protect the respondents from incurring abuse as a result of responding to these questions. Their husbands will not be asked any questions about domestic violence, nor will they be told if their wife does report a history of violence and the couple is disqualified from the study. Thus, we will follow current WHO ethical and

safety recommendations for research on domestic violence against women (www.who.int/violence_injury_prevention/vaw/ethicsenglish.htm) The counselors will de-brief the women at the end of the survey, giving them ready responses for husbands or other family members who inquire about the nature of the questions.

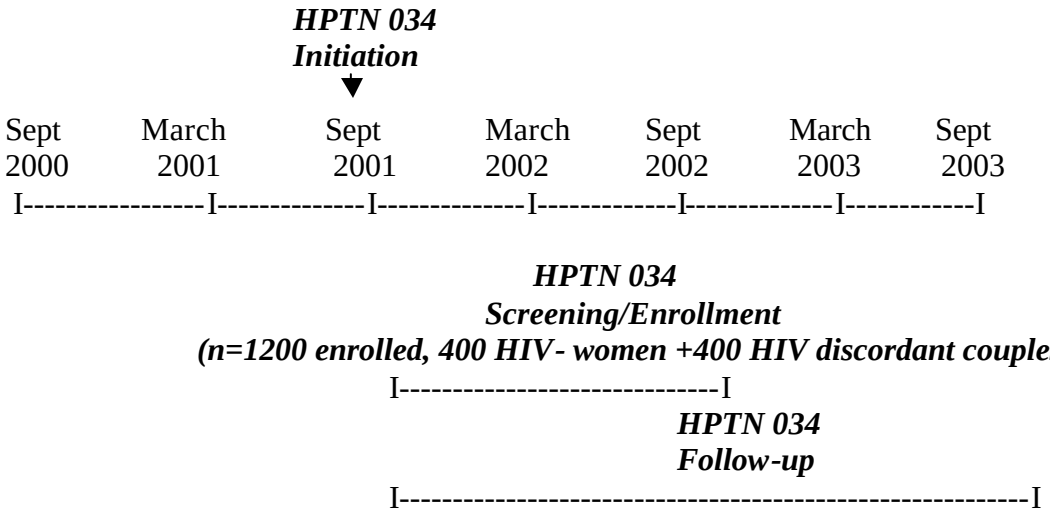
3.5 Withdrawal

Once a participant has enrolled in the cohort study, we will make every reasonable effort to retain him/her for at least 12 months of follow-up. Retention rates of at least 95% are targeted for this observational cohort study. However, participants will have a right to withdraw from the study for any reason at any time if they so desire. The investigator also may withdraw participants from the study in order to protect their safety and/or if they are unwilling or unable to comply with required study procedures. Participants also may be withdrawn if the sponsor or regulatory authorities terminate the study prior to its planned end date.

4 STUDY PROCEDURES

The objective of our efforts will be to ensure the availability of a sufficiently large cohort of high-risk men and women for participation in the multiple HPTN clinical trials, with incident HIV infection as the primary outcome measure. Our goal will be to continue to screen 2000 new male and female high-risk patients/year, with an expected HIV prevalence of 20%. Of the 1600 HIV-uninfected individuals, we will anticipate enrollment of approximately 400 new non-sex worker women over the first 12 months into this follow-up study of HIV seroconversion. We will also enroll 400 HIV-discordant couples. The estimated study time line is outlined in the chart below.

HPTN 034 Timeline



4.1 Screening

We will continue to inform all new STD patients that we are offering a free, voluntary HIV test and we will examine and treat the patient for an STD or other infections or medical problems (See Section 4.6 below). For this study, our standard syndromic STD diagnosis and treatment procedures (for screening and follow-up) will be followed.

Men and women with symptoms and/or signs of a genital ulcer disease will be offered one of the following 4 syndromic treatment options by the clinicians:

A: Inj. Ceftriaxine 250 mg IM and Cap. Doxycycline 100 mg BID*14

OR

B: Inj. Ceftriaxone 250 mg IM and Tab Azithromycin 1 gm stat

OR

C: Cap Doxycycline 100 mg BID*14 and Tab Ciprofloxacin 500 mg BID*3

OR

D: Tab Erythromycin 500 mg QID*15 [Pregnant women]

Men and women with symptoms and/or signs of genital discharge or urethritis/ cercicitis/ vaginitis will be offered one of the following 2 syndromic treatment options by the clinicians:

A: Inj. Ceftriaxone 125 mg IM and Cap Doxycycline 100 mg BID*14

OR

B: Ceftriaxone 125 mg IM and Tab Azithromycin 1 gm stat

AND

- 1 Tab Metronidazole 2 gm stat for Trichomonas
- 1 Tab Metronidazole 400 mg BID*7 OR Secnidazole 2 gm stat for bacterial vaginosis [BV]
- 2% Myconazole *7 OR Candid Vaginal Tablets V1 (1 day) or V6 (6 days) for vaginal candidiasis

If the patient refuses a HIV screening, we will treat the patient based on physical signs and symptoms, and invite them to come back any time for a free HIV test. If the patient agrees to have an HIV test, consent will be obtained, the patient will be examined by the doctor, blood will

be drawn and the patient will be asked to come back in approximately 7 days for the test results. Patients will also be offered free screening for syphilis. A short screening questionnaire will be administered to collect basic demographic and risk behavior data. As part of our pre-test counseling, all patients will be given a short oral presentation on the modes of acquiring HIV, correct use of condoms will be demonstrated using an anatomical model, and patients will be given as many condoms as they request.

4.2 Enrollment

When people return for their test results and post-test counseling (typically within 1 week of their screening visit), individuals will be informed that we are doing a study at the clinic and they will be invited to join the observational follow-up study. The requirements of this observational study (follow-up for 1 years, phlebotomy, physical exams and questionnaires) are designed to prepare the participants and project staff for HPTN 035 and 052 clinical trial protocols. Of the 800 HIV-uninfected participants targeted for enrollment, 400 will be enrolled with their HIV-infected sexual partners. These 400 HIV-discordant couples will be consented, enrolled and followed as couples, using identical data forms and procedures (Note exception: HIV-infected partners will not have repeat HIV testing) as for the 400 HIV-uninfected women in this study. The procedures for participation in the cohort study will be thoroughly explained to each patient and couple. We will also inform the participants that we are preparing for some studies of new methods for preventing HIV infection in the near future.

Enrollment and recruitment will be done only by clinic counselors and physicians, who will answer all questions posed by patients. If the patient refuses to join the study at that time, they will be told that they can still return any time for medical care or to join the study at a later point. In addition, we will collect information about the reasons for their refusal to participate in the cohort study. Informed consent will be obtained from eligible and willing participants for all HPTN studies.

4.3 Follow-up Visits

Follow-up visit will occur every 3 months for 1 year. At every follow-up visit, a short questionnaire will be administered to all participants to assess their continued understanding from previous visits about the nature, duration, benefits and risks of being in the study and to assure their voluntary participation in the study. Patients seen in follow-up will also have a physical examination, be evaluated and treated for STDs, receive additional counseling and offered re-testing for HIV infection as outlined below. In addition, participants will also be invited to participate in additional assessments issues addressing retention using a series of focus groups, key informant interviews and individual qualitative and quantitative assessments. At each follow-up visit, we will also utilize exit interviews, focus groups and semi-quantitative methods to assess the factors identified at screening that are associated with retention and/or loss to follow-up in these newly enrolled individuals.

4.4 Detection of HIV infection

For this HPTU protocol, identification of incident cases of HIV-1 infection will be primary outcome measure. Sera will be initially screened with the Genetic Systems combination ELISA kit for detection of HIV-1 and HIV-2, which is our current ELISA screening method. Specimens that are positive by Genetic Systems ELISA will be confirmed for HIV-1 or HIV-2 infection by re-testing with a different ELISA (UBI HIV 1/2 EIA, United Biomedical, USA). All HIV testing will be performed on site in Pune. The HPTN Central Lab will implement and monitor the quality control procedures for all of the laboratory assays for this protocol.

4.5 Interim Contacts and Visits

Interim contacts and visits may be conducted at participant request at any time during the study. Interim HIV counseling and testing will be provided as needed in response to participant reports of potential exposure to HIV. All interim contacts and visits will be documented in participants study records and on applicable case report forms.

4.6 Clinical Care for Study Participants

For this protocol we will continue our current standard of clinical care, which is to provide free diagnosis and treatment of all STDs, HIV VCT, as well as referral to the NARI TB clinic, the NARI HIV Clinic or to the many specialty clinics attached to Sassoon Hospital, if necessary. This clinical care package is offered to all walk-in patients in our clinics, whether or not they are enrolled in research studies. In addition the standard of clinical care for all patients attending the NARI clinics exceeds what is locally available to most patients with and without HIV infection. All outpatient clinical care costs, including the cost of most medications for STDs, opportunistic infections, TB and other common illnesses will continue to be provided free of charge to all participants. Specific clinical diagnostic testing and treatment to be provided to study participants and to those screened for possible study participation are outlined in detail in Section 4.1 above and 4.7. below.

Currently, anti-retroviral therapy is affordable to less than 5% of the HIV-infected patients in India. The cost of these medications has recently been significantly reduced by some companies in India (e.g. CIPLA). Despite these reductions, the medications will still cost an estimated $600-$1000/person per year, which is more than the yearly income of most of the HIV-infected individuals coming to the NARI clinics for HIV screening and STD treatment. In addition, there have been no clinical trials in India to measure the clinical efficacy of HAART. The impact of HAART for prevention of the most common causes of morbidity and mortality in India (TB, herpes zoster, neurological disease, bacterial pneumonia, diarrhea, etc) has not been measured or compared with the impact of other interventions, such as INH prophylaxis, cotrimoxazole prophylaxis and provision of adequate nutrition and clean drinking water. It is also unclear if the current North American guidelines for initiation of HAART (CD4<350) are optimal for Indian patients who are at great risk of HIV-associated death from diseases that occur at higher CD4 counts. A HPTN network wide policy is required for all protocols to define what should be the minimal level of care provided to all HPTN participants. In the absence of a policy and/or HPTN support for provision of HAART to all participants, we will continue to provide care that

is consistent with Indian government guidelines (National AIDS Control Organization and Indian Council of Medical Research). The Indian government currently does not require provision of HAART to HIV-infected participants in clinical trials.

HIV-infected participating in this cohort study, either index partners of a discordant couple or as a participant in the laboratory substudy (HPTN 034A) of recently infected persons will be offered the following additional care for the duration of this study (Section 4.6.1 and 4.6.2). HIV-infected individuals found at screening or seroconverters unwilling to participate in the 034A lab substudy will be offered referral to the NARI HIV clinic or to a local provider for clinical care.

4.6.1. All HIV-infected Enrolled Study Participants

- CBC and CD4 count every 3 months (Note: subjects enrolled in 034A substudy will follow schedule outlined in appendix for CD4 monitoring)

- HIV viral load every 6 months (Note: subjects enrolled in 034A substudy will follow schedule outlined in appendix for viral load monitoring)

4.6.2. Symptomatic (or CD4<200) HIV-infected Enrolled Study Participants

- Cotrimoxazole prophylaxis (Based on UNAIDS guidelines for sub-Saharan Africa)

4.6.3. HIV-infected Persons identified at Screening or HIV seroconverters not participating in the HPTN 034A Laboratory Sub-study

- Referral to Indian Government supported NARI HIV clinic: This clinic provides free counseling CD4 counts every 6 months, yearly viral load, co-trimoxazole prophylaxis, multivitamins, medical care for specific illnesses as per the national standards of care and referrals to specialty care at the local government hospital, when required.

- Patients will also be offered option of referral to local physicians experienced in the care of HIV-infected individuals.

4.7 Ancillary HPTN Core Laboratory Studies

Participants in this 034 protocol may also be eligible to enroll into one of two proposed ancillary laboratory sub-studies (Acute HIV Study 034A and Exposed-Uninfected Study 034B), directed by the HPTN Core Laboratory. Details of these sub-studies are included in the appendix. In addition, to prepare the site for upcoming trials and to establish required QC for microbiological assays that will be required, some additional laboratory tests to diagnose the etiology of STDs will be undertaken in symptomatic STD screened and enrolled subjects. Although, as mentioned above, treatment of screened and followed patients will follow a syndromic algorithm, the following additional STD laboratory assays will be performed when clinically indicated:

4.7.1. STD Diagnostic Procedures Offered to All Women Presenting to the STD Clinics

All women presenting to the STD clinics in Pune will be routinely offered a pelvic examination that includes bimanual examination for adnexal masses or tenderness and speculum examination of vagina and cervix. The following STD diagnostic algorithm will be followed:

- **IF** abnormal vaginal discharge or purulent cervicitis is noted:
    - Collect specimen for wet mount for candida, trichomoniasis, and BV (pH and Gram stain).
    - Collect swab for *N. gonorrhoeae* and *C. trachomatis*

- **IF** ulcerative lesions are noted:
    - Collect swab for syphilis dark field microscopy and collect blood for syphilis serology.

4.7.2 STD Diagnostic Procedures Offered to All Men Presenting to the STD Clinics

All men presenting to the STD clinics in Pune will be routinely offered a physical examination which includes a genital examination. The following algorithm will be followed:

- **IF** genital discharge by report or examination is noted:
    - Collect specimen for wet mount for candida, trichomoniasis.
    - Collect swab for *N. gonorrhoeae* and *C. trachomatis*

- **IF** ulcerative lesions are reported or examination is noted:
    - Collect swab for syphilis dark field microscopy and collect blood for syphilis serology.

4.7.3. Additional Protocol Specific STD Laboratory Procedures

Details of the STD laboratory Protocol specific procedures are pending from the HPTN STD Core Laboratory (T. Quinn, Director). Some additional laboratory procedures may be utilized in this protocol for participants in the cohort study, in anticipation of what will be required for the 035 and 052 protocols. The purpose of introducing these assays in this protocol will be to assist in the establishment of QC procedures with the HPTN STC Core Laboratory. These additional laboratory assays will be phased in to reflect final decisions about what assays will actually be utilized for the 035 and 052 trials. These may include:

- Examine InPouch culture for *T. vaginalis.*
- Conduct PCR for *N. gonorrhoeae* and *C. trachomatis*.
- Serology for HSV-2

4.8 Assessment of Barriers to Trial Participation and Retention

To prepare for upcoming HPTN trials, we need to explore attitudes and experiences regarding participation in clinical trials. We must thoroughly understand acceptance of HIV prevention research and willingness to participate in research and research trials. The constructs of 'clinical trials', 'randomization', 'placebo' etc. will need to be understood by potential research subjects. Exploring the cultural differences between researchers and potential subjects regarding these constructs will provide new information, enlighten research design and inform foundational ethical principles for upcoming trials. We will need to understand the local terms or concepts that capture these constructs so they can be incorporated into recruitment and informed consent strategies for HPTN trials. We will explore the kinds of information needed by potential study subjects and how they would like that information presented (flip charts, videos, verbal, written) and by whom (peer counselors, nurses, doctors). Lastly, we will explore people's reaction to the idea of biomedical research itself. We will conduct qualitative in-depth and focus groups methods to explore these kinds of questions like: Is it right for a doctor to try a new or untested therapy on a patient in order to see what happens? How is knowledge acquired in medical systems in India, i.e. Ayurvedic, allopathic or other? Who should benefit from research, participants, society or both? Who would you talk to, in order to decide to be in a research study? Why? What ways do you usually get new information? How do you prefer to get information on new problems, issues, or products? How do you usually make a decision about how to solve a problem? What is 'research'; how would you describe 'informed consent'? What local terms would be appropriate? Why would you enroll in a research study? Why not? What kinds of incentives would be appropriate for people to enroll in a study?

The qualitative research plan consists of a triangulation of ethnography, focus group discussions, and indepth interviews of potential participants. Focus group guides have been developed for HIVNET 013, and will be used as the basis for FGD by trained facilitators. These guides are standardized, and outline the specific topic areas for data collection and elaboration. In addition, standardized guides will be developed for in depth interviews. Transcripts from FGD and indepth interviews will be reviewed for completeness, entered into a database verbatim, reviewed for topical areas and coded in the local language, and analyzed. Informants for in-depth interviews and focus group discussions will be purposively sampled based on the following criteria: HIV-negative, native Marathi or Hindi speaker, currently involved in research at the clinic or recently experienced the research process at the clinic and are thus knowledgeable about the topic of interest, able and willing to discuss issues related to research participation and procedures.

4.8.1. Qualitative interviews

At the enrollment visit, a subset of 15-18 HIV negative eligible women and 20-25 eligible couples will be purposively selected and invited for in-depth qualitative interviews (Eligibility is defined as those who meet the criteria for enrollment as described in section 3.3 of the protocol). Half of the sample of in-depth respondents will be obtained from those who refuse to enroll in the study, to assess their concerns about study participation and their understanding and opinions about research and clinical trials.

4.8.2. Focus groups

4-6 focus group discussions will be conducted, each with 6-8 participants. These will be selected purposively from those women and couples who are eligible to participate in the cohort and who are willing to participate in the focus group. They will be selected at the enrollment visit. In-depth interviews and focus group discussions will be conducted by a pair of trained interviewers and facilitators. They will be conducted in Hindi / Marathi (local languages).

4.8.3. Qualitative Data Collection and Analyses

Where focus group discussion participants and in-depth interview participants are willing, tape recorders will be used. Field notes will be expanded and tapes will be transcribed and translated into English. Data will be analyzed using Nudist or AtlasTi software for ethnographic data according to the following themes:

- Participant / Respondent Understanding of the term "research"
- Understanding the term "informed consent"
- Understanding of the term "randomization"
- Understanding of the term "placebo"
- What are the barriers to participation
- Categories of the different types of barriers to participation (e.g personal – family, husband or partner refuses permission, work, stigma)
- Attitudes / beliefs towards participation in research trials

These themes will be further compared across gender, those who are willing to participate versus those who are not, levels of education and age. The analysis of the qualitative data will focus on those barriers to participation and retention that can be addressed at the clinic level and through modification in the study or project protocols (e.g. using pictorial charts to explain clinic procedures simply and clearly) and those that cannot be addressed at the clinic level (e.g. inability to participate because loss of wages/work). Input from the community advisory board will be sought to develop ways to reduce possible costs (e.g. child care, transportation, food) to those enrolled in the study. For HIV negative women participants, efforts will be made to understand and address whether their participation in the study will have negative consequences (i.e. violence, stigma) in their relationships with spouses and families.

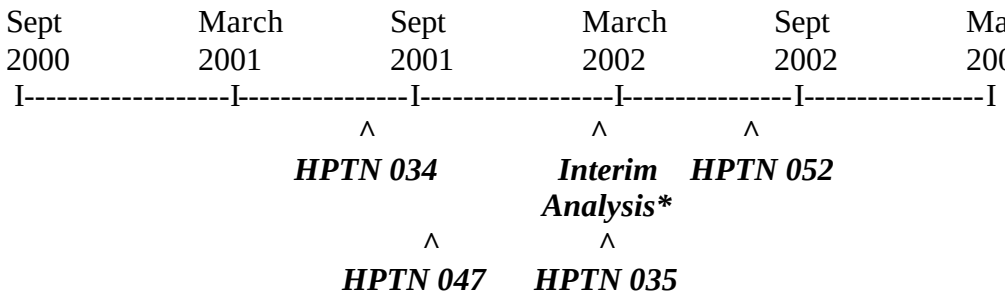
4.9 Transition from Preparedness Studies to HPTN Clinical Trial Implementation

While these preparation studies are underway, we will also be participating in the final development and planning for the HPTN 035 and 052 trials planned for the site in Pune. Therefore, we require a plan for transitioning from preparedness work to protocol implementation. We propose that the HPTN 034 protocol team undertake an interim analysis of the primary endpoints of this protocol, when the final protocols for the Phase III microbicide trial and the ART trial are submitted to the PSRC for review. Based upon the results of these analyses

and the time-lines for implementation of the clinical trials, the protocol team in consultation with the EC and PRC will determine whether the activities focused upon addressing any of the primary endpoints will be terminated and resources shifted towards plans for trial implementation.

There are 2 Phase III trials (035 and 052) and 1 Phase I/II trial (047) planned for this site in Pune. We have conducted multiple large simultaneous cohort studies and phase I clinical trials in the past. However, we must plan the conduct of this preparation study and the initiation of these clinical trials carefully to ensure the successful implementation and coordination of these activities and our resources. It is our expectation that the timing of the initiation of the subsequent HPTN clinical trials in Pune will be carefully coordinated. For both of the proposed phase III trials, the Pune site is proposed as one site in a multi-centered trial. Given the time and effort required to finalize and review each of these protocols within the HPTN, NIAID, IRBs and host countries, we do not anticipate that the trials will be initiated simultaneously. However, with planned and coordinated initiation of these trials, we do have the capacity for simultaneous enrollment and follow-up for multiple HPTN clinical trials. In addition, we have selected the trials for participation carefully. Each trial, as currently planned, will recruit distinct subjects from our clinics. Our current estimated timeline for the activities in Pune, based on the status of each of the trial protocols is as follows:

Pune Projected HPTN Protocol Initiation Timeline



Interim HPTN 034 Analysis

5 STATISTICAL CONSIDERATIONS

5.1 Primary Endpoints

- Current HIV seroconversion rates in high-risk men and women.
- Retention rates in high-risk men and women, as well as discordant couples.

5.2 Secondary Endpoints

- High-risk behaviors for HIV and STDs in newly enrolled participants.

  A number of secondary risk factor endpoints will be assessed, including:

  - Number of sex partners.
  - Presence and history of genital ulcer disease, urethritis and cervicitis.
  - Presence of circumcision.
  - Frequency of vaginal intercourse.
  - Frequency of unprotected vaginal intercourse.
  - Frequency of anal intercourse.
  - Frequency of unprotected anal intercourse.
  - Frequency of injection drug use.
  - Frequency of sharing injection drug equipment
  - Frequency of sharing injection drug equipment without cleaning.
  - Frequency of sexually transmitted diseases.
  - Frequency of serologic evidence of syphilis infection.

- Effective strategies to retain participants and overcome barriers to participation for upcoming HPTN clinical trials

  As outlined below, we will calculate 6 and 12 month retention rates for the 400 newly identified and enrolled HIV uninfected women, as well as the 400 HIV discordant couples. We will also assess the factors identified at screening and follow-up that are associated with retention and/or loss to follow-up in these newly enrolled individuals. The in-depth interviews and focus groups will address a number of key questions a) what are the barriers to their participation in the studies; b) what do they think can be done to address their difficulties or obstacles to participation in the studies; c) What would motivate them to participate; d) what would motivate them to come back or continue with this study (this question is for those who have already enrolled and are coming for their follow up visits).

5.3 Sample Size Considerations

5.3.1 HIV Seroincidence

Our sample size is based on the number of new high-risk patients required to be enrolled over the next year to provide an estimate of HIV incidence. Separate HIV seroincidence rate will be computed for women and HIV-uninfected partners in our cohort study as the total number of HIV seroconversions divided by the total number of person-years of follow-up. Approximately 2000 new STD patients will be screened over the next year and 400 newly identified HIV-uninfected high-risk women and 400 HIV serodiscordant couples attending the STD clinics in Pune will be enrolled in order to estimate the current HIV incidence. Based on recent experience in our study, those participants screened 1999-2000 had a HIV prevalence rate of 19.0%. The overall HIV incidence in our cohort of initially HIV-uninfected individuals followed from May 1993 through April 2000 was

5.9/100 person years (Men=5.2/100PY, Women=9.8/100PY). Based upon the most recent experience of our study, the overall HIV incidence among those enrolled in the 1997-1999 cohort of HIV-uninfected individuals was 4.2/100 person years (Men=3.9/100PY, Women 5.6/100PY).

The null seroincidence used for sample size calculations will be the recent estimate of seroincidence in our cohort. This 034 protocol will enroll 400 women, as well as 400 HIV-uninfected partners of HIV-infected persons and follow them for 1 year, with an anticipated 90% semiannual retention.

The 400 high-risk women participants will accrue an estimated 348 person years of follow-up by July 2003. The study sample size will be adequate to estimate a 95% confidence interval for HIV incidence in the range of 3.37-8.74/100PY, assuming expected rates equal to those recently experienced in the cohort.

Amongst the discordant couples, the 400 HIV-uninfected partners will accrue 348 person years of follow-up, which will be sufficient to estimate a 95% confidence interval for seroincidence of 8.46-15.93/100PY, assuming an expected rate of 11.8/100PY (Quinn TC et al, NEJM 2000, 30;342(13):921-9. Note: there are no estimates of discordant couple transmission rates available for India).

The assumption used provides a conservative sample size estimate. While the target rate is 95% annual retention, the actual recent experience of the cohort is 81% semiannual retention. The assumption used in the sample size calculations of 90% semiannual retention will assure adequate power to measure a minimal level of incidence in the event that the target retention is not achieved.

[Note: Our site has successfully managed an average of 311 study visits per month over the past 8 years (median 305.5, range 150-475 visits per month), including screening, enrollment and follow-up visits. The present study will require 233 - 414 visits per month in the first year, and 54 - 234 visits per month in the second year. While the clinics will be operating at full capacity for the second half of year one, the workload will not overwhelm staff capabilities because half of the expected number of visits will consist of follow-up visits, which require considerably less staff time to complete than screening or enrollment visits]

5.3.2 Study Retention Rates

Amongst the 400 HIV-negative women enrolled in the study, we are targeting a 95% annual retention rate. Using that expected rate, the sample size of 400 will allow the estimation of the retention rate to within $\pm$ 2.14% (95%CI). If the estimated retention were to drop to 90% the associated 95%CI is $\pm$ 3.0%, and if it were to drop as low as 80%, the sample size would allow the calculation of a 95% CI of $\pm$ 3.9%. Similarly, amongst the 400 HIV-uninfected members of serodiscordant couples we estimate that we will achieve 95% annual retention. This number of couples will allow for a 95% confidence interval of $\pm$ 2.14%, and for 90% retention the interval increases to $\pm$ 3.0%.

5.4 Data Monitoring and Analysis

Close cooperation between the study site Investigator, Protocol Coordinator, Biostatistician, Data Managers, and other study team members will be necessary in order to track study progress, respond to queries about proper study implementation, and address other issues in a timely manner. Rates of accrual, follow-up, and protocol compliance will be monitored closely by the study team. Representatives of the HPTN CORE and SDMC also will evaluate these rates on a regular basis. If unexpected concerns arise, they will be addressed according to HPTN standard operating procedures.

The two primary objectives of this protocol will be:

- To determine the current incidence of HIV among a cohort of 400 newly identified high-risk, HIV-negative women in Pune. In addition, a separate estimate of the HIV incidence among 400 HIV-uninfected partners of HIV-infected subjects will also be estimated separately.

- To determine the retention rate in high-risk women, as well as HIV discordant couples.

Corresponding to each of the primary study objectives and outcomes, the following primary data analyses will be performed:

- An HIV seroincidence (and 95% confidence intervals) for the cohort study will be computed as the total number of HIV seroconversions divided by the total number of person-years of follow-up. Separate estimates of seroincidence will be calculated for the 400 women and the 400 HIV-uninfected partners.

- The number of enrolled participants retained for each study follow-up interval will be tabulated. Retention rates also will be calculated. The denominator for these calculations will be the total number of participants enrolled in the study. The numerator will include all participants who complete a Follow-up Pre-Test Visit during the interval and/or are known to have become HIV-infected or to have died during a previous interval. Overall retention rates will be calculated for all 1200 subjects. In addition, the retention rates of the subset of 400 women and 400 discordant couples will be separately calculated.

- Univariate and multivariate analysis will be performed for risk factors for incident HIV infection.

Qualitative data analysis: In addition, corresponding to secondary study objectives, the HIV risk behaviors and assessment of barriers to enrollment and retention of study participants, in both the cohort and the community study will be described. In order to prepare for possible participation in the HPTN 052 trial, we will utilize qualitative and quantitative social science techniques to identify and address particular behavioral and social barriers to enrollment and retention of HIV discordant couples. In addition, the rate

of participant retention for each study follow-up interval, and across all four study visits, will be calculated. The baseline demographics and HIV risk behaviors of women and couples completing and not completing scheduled follow-up will be compared.

6 HUMAN SUBJECTS CONSIDERATIONS

6.1 Ethical Review

This protocol has been reviewed and approved by the local NARI Ethical Committee, the Central Ethical Committee of the Indian Council of Medical Research and the Johns Hopkins University JCCI (IRB). A Cooperative Project Assurance has been filed by the local NARI EC and has been approved by the OHRP of the USPHS. All attached consent forms have received preliminary review and approval by both the in-country IRB and the JHU JCCI. In order to prepare for the specific HPTN trials outlined in this study, we have submitted for review modifications to these previously approved consent forms addressing the issues of identifying and enrolling HIV discordant couples. The modified forms are attached in Appendix II and are under review by the local IRB and JHU JCCI. These modified consent forms will not be used until final approval is obtained from both of these ethical committees.

The local IRB/EC and the JHU JCCI will review this protocol at least annually. Dr. Mehendale will make safety and progress reports to the NARI IRB/EC at least annually, and within three months of study termination or completion. These reports will include the total number of participants enrolled in the study, the number of participants who completed the study, all changes in the research activity, and all unanticipated problems involving risks to human subjects or others.

6.2 Informed Consent

Written informed consent will be obtained from each study participant. The informed consent forms that we utilize (attached) have been developed for local use, translated/back-translated in 2 local languages (Hindi and Marathi) and describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation, in accordance with all applicable regulations. Participants will be provided with a copy of their informed consent form if they are willing to receive them. Study staff will document the informed consent process as described in the study-specific procedures manual.

6.3 Confidentiality

All study-related information will be stored securely at the study site. All participant information will be stored in locked file cabinets in areas with limited access. All laboratory specimens, reports, study data collection, process, and administrative forms will be identified by a coded number only to maintain participant confidentiality. All records that contain names or other personal identifiers, such as locator forms and informed consent forms, will be stored separately from study records identified by code

number. All local databases will be secured with password-protected access systems. Forms, lists, logbooks, appointment books, and any other listings that link participant ID numbers to other identifying information will be stored in a separate, locked file in an area with limited access. Participants' study information will not be released without the written permission of the participant, except as necessary for monitoring by NIAID and/or its contractors (e.g., the DAIDS monitoring contractor), representatives of the HPTN CORE and/or SDMC, and/or the Indian Council of Medical Research (ICMR).

6.4 Risks

Some people get sore at the place where blood is drawn, Some people get dizzy or feel faint when blood is drawn. It is possible that participation in this study could result in others outside of the research project learning of the participants HIV status. Research staff will be trained and admonished not to reveal any information to anyone not directly associated with the study. The inclusion of a sub-set of HIV discordant couples in follow-up, interviews and group interviews may cause some unease due to worries or concerns about their risks or due to disagreements with their partner in joint interviews. If the level of discomfort becomes severe, additional counseling for both individuals and couples, will be provided by staff or a referral will be made as needed in such cases. There is also the risk that participants in group interviews will be concerned that other participants in the group will tell others outside of the group their identities and/or what has happened in the group. In the groups, a discussion of the needs for confidentiality for all participants will be held to avoid this to the extent possible. During group discussions, pseudonyms will be used in order to protect the identity of the participants.

6.5 Incentives

The local NARI EC has approved the procedures for compensating participants for their time and effort in this study, as described in the consent forms. Consistent with local standards of practice, participants will receive compensation in the form of cash at each study visit, to reimburse for the costs of lost work, travel, and/or child care associated with study visits.

6.6 Communicable Disease Reporting Requirements

There is no requirement for reporting the diagnosis of STDs and HIV among study participants in India.

6.7 Study Discontinuation

The study may be discontinued at any time by the ICMR, NIAID, the HPTN, and Indian or US regulatory authorities.

7 LABORATORY SPECIMENS AND BIOHAZARD CONTAINMENT

As the transmission of HIV and other blood-borne pathogens can occur through contact with contaminated needles, blood, and blood products, appropriate blood and secretion precautions will be employed by all personnel in the drawing of blood and shipping and handling of all specimens for this study, as currently recommended by the US Centers for Disease Control and Prevention.

8 ADMINISTRATIVE PROCEDURES

8.1 Study Coordination

Study implementation at all sites will be directed by this protocol as well as a site-specific procedures manual. This manual will outline procedures for conducting study visits, collecting and submitting study data, and other study operations. Data will be collected on site in India using existing data forms which will be modified to incorporate specific study procedures outlined in this protocol. Our data management team will work with the HPTN SC to develop data management procedures for collecting the data and quality control monitoring. The goal is to put into place data procedures and coordination with the SC for this HPTN 034 study that will facilitate rapid transition to the proposed HPTN clinical trials for Pune.

8.2 Study Monitoring

On-site study monitoring will be performed in accordance with HPTN policies. Study monitors will visit the site to verify compliance with human subjects and other research regulations and guidelines; assess adherence to the study protocol, study-specific procedures manual, and locally-accepted HIV counseling practices; and confirm the quality and accuracy of information collected at the study site and entered into the study database. The site investigator will allow study monitors officials to inspect study facilities and documentation (e.g., informed consent forms, clinic and laboratory records, other source documents, case report forms, product accountability forms), as well as observe the performance of study procedures. The investigator also will allow inspection of all study-related documentation by authorized representatives of the ICMR, HPTN CORE, SDMC, NIAID, and US and in-country regulatory authorities. A site visit log will be maintained at the study site to document all visits.

8.3 Protocol Compliance

The study will be conducted in full compliance with the protocol. With the exception of modifications required to eliminate immediate participant safety concerns, the protocol will not be amended without prior written approval by the Protocol Chair or designee; all protocol amendments will be submitted to and approved by the NARI and Hopkins ethical committees, as well as the HPTN CORE prior to implementing the amendment.

8.4 Investigator's Records

The study site investigator will maintain, and store in a secure manner, complete, accurate, and current study records throughout the study. The investigator will retain all study records for at least five years after the completion of the study, unless directed otherwise by the HPTN CORE. Study records include administrative documentation — including site registration documents and all reports and correspondence relating to the study — as well as documentation related to each participant screened and/or enrolled in the study — including informed consent forms, locator forms, case report forms, notations of all contacts with the participant, and all other source documents.

8.5 Use of Information and Publications

Publication of the results of this study will be governed by DAIDS and ICMR policies. Any presentation, abstract, or manuscript will be made available by the investigator to the HPTN Manuscript Review Committee, and DAIDS for review prior to submission.

Appendix I

HPTN HIV Prevention Preparedness Study Protocol
Pune, India

SCHEDULE OF EVENTS
COHORT STUDY

PROCEDURE	Screening Visit	Enrollment Visit	Follow-up Visits[§§]
Obtain informed consent[#]	X	X	X
Collect demographic information	X		
Determine eligibility[§]	X	X	
Administer risk assessment	X	X	X
Deliver HIV counseling	X	X	X
Provide contact information and instructions	X	X	X
Physical exam, clinical diagnosis, treatment and referral services	X	X	X
Complete and submit data collection forms	X	X	X
HIV Antibody Test	X		X
Syphilis Test	X		X
DX and Treatment of STDs[##]	X	X	X

[#]In addition to consent for HIV screening and enrollment, participants who participate in the focus groups, in-depth interviews and/or laboratory sub-studies will have a separate consent obtained.

[§]Demographic and behavioral eligibility is determined and documented at the Screening Visit (e.g. lack of psychological or emotional factors that would make them ineligible. Also, women must have not reported history of commercial sex work to be eligible for enrollment in the female cohort); eligibility related to HIV status is determined and documented at the Enrollment Visit. In addition, HIV-infected individuals are assessed for eligibility for enrolling as part of discordant couples cohort (i.e. a sexual partner with regular sexual contact over prior 3 months)

[##]Participants will be evaluated and treated for STDs at any scheduled visit or PRN.

[§§]Follow-up visits will occur every 3 months for 1 year

Appendix II

HPTN 034: HIV Prevention Preparedness Study Protocol
Pune, India

Proposed Consent Forms

Appendix III

HPTN 034A: HIV Prevention Preparedness Study Protocol
Pune, India

HPTN 034A Early Infection Laboratory Sub-study

HPTN 034 HIV Incidence and Participant Retention Protocol

HPTN EARLY HIV-1 INFECTION
HPTN 034A LABORATORY SUB-STUDY

THE HPTN CENTRAL LABORATORY

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Dr. Madhur Thakur
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  - Addendum 2 Consent for Future Use of Blood Samples
- 034A Sample Informed Consents for Seronegatives
  - Addendum 1 Consent for Future Use of Blood Samples

Appendix A: Table 1: Visit Schedule for Seropositives
Appendix B: Table 2: Visit Schedule for Seronegatives
Appendix C Viral Load Measurement Protocol
Appendix D PBMC Isolation and Cryopreservation /ELISPOT Assay Protocol

ABBREVIATIONS

Ab	antibody
AACTG	Adult AIDS Clinical Trial Group
AIDS	acquired immune deficiency syndrome
bDNA	branched deoxyribonucleic acid
CL	Core Laboratory
CD	cluster designation
CDC	Centers for Disease Control and Prevention
CM	culture medium
CTL	cytolytic T lymphocyte
env	envelope
FHI	Family Health International
FBS	fetal bovine serum
FACS	fluorescent activated cell sorter
FM	freezing medium
gag	group associated antigen
gp	glycoprotein
HCL	HPTN Central Laboratory
HIV-1	human immunodeficiency virus type 1
HIVNET	HIV Network for Prevention Trials
HLA	Human Leukocyte antigen
HPTN	HIV Prevention Trials Network
HTPU	HIV Prevention Trials Unit
IFNγ	interferon-gamma
MHC	major histocompatibility complex
min	minutes
ml	milliliter
MRC	Medical Research Council
Nef	negative factor
NIAID	National Institute of Allergy and Infectious Diseases
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PHA	phytohemaglutinin
RT	reverse transcriptase
STD	sexually transmitted disease
WM	wash medium

HPTN 034A
HPTN EARLY HIV-1 INFECTION LABORATORY SUB-STUDY

A Study of the HIV Prevention Trials Network

Sponsored by:

Division of AIDS (DAIDS)
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US National Institutes of Health (NIH)

We, the Principal Investigators, agree to conduct this study in full accordance with the provisions of this protocol. I agree to maintain all study documentation for a minimum of five years from the end of the study, unless directed otherwise by the HPTN Coordinating and Operations Center (CORE). Publication of the results of this study will be governed by HPTN and DAIDS policies. Any presentation, abstract, or manuscript will be made available by the investigators to the HPTN Manuscript Review Committee and DAIDS for review prior to submission.

We have read and understand the information in this protocol and will ensure that all associates, colleagues, and employees assisting in the conduct of the study are informed about the obligations incurred by their contribution to the study.

Name of Principal Investigator _____

_____ _____
Signature of Principal Investigator Date

Name of Principal Investigator _____

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Signature of Principal Investigator Date

HPTN 034 A
HPTN Early HIV-1 Infection Laboratory Sub-Study
PROTOCOL SUMMARY

Design:	Laboratory Sub-study for HPTN 034
Population:	Adult men and women who have been identified as recently infected (within prior six months), enrolled in HPTN 034.
Study Duration:	This sub-study is designed to run for the duration of entire HPTN 034 funding period. Participants will be continuously enrolled and followed for up to two years.
Primary Objectives:	**(a)** To compare initial HIV-1-specific CD8-mediated immune response with HIV-1 viral set point. **(b)** To compare host immunologic genetic characteristics with HIV-1 viral set point.
Primary Endpoints	**(a)** Median HIV-1 viral load between 6 and 12 months after estimated time of primary infection. **(b)** ELISPOT measurement of HIV-1 peptide-specific T lymphocytes **(c)** HLA-Class I MHC alleles.
Secondary Objectives:	**(a)** To characterize the epitope specificity of the initial CD8-mediated immune response. **(b)** To provide immunological and virological laboratory training and to establish laboratory quality control procedures for the NARI HPTU. **(c)** To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples. **(d)** To establish a repository of plasma and PBMC specimens from recently HIV-1-infected individuals, for future HPTN and collaborative immunologic and virologic studies.
Secondary Endpoints:	**(a)** Characterization of MHC Class I restriction and minimal HIV-1 peptide sequence of viral-specific T lymphocytes in early HIV-1 infection. **(b)** Quality control assessment of HIV-1 viral load and ELISPOT assays for site in Pune. **(c)** Measurement of enrollment and retention of study participants (and their primary partners, if enrolled in the HPTN 034B Exposed-Uninfected Sub-study).

1.0 INTRODUCTION

This small laboratory sub-study is proposed for HPTN 034 in Pune, India. In addition, procedures from this protocol will be proposed for sub-studies of number of other HPTN trials, that have new HIV infection as a primary outcome measure. It is anticipated that in addition to descriptive analysis of site-specific laboratory data from HPTN 034 in Pune, consistent implementation of procedures and schedules of this 034A protocol for other HPTN protocols will provide a unique opportunity to compare results from multiple protocols and to consider a meta-analysis in the future.

1.1 Background

AIDS, caused by infection with the human immunodeficiency virus 1 (HIV-1), is associated with enormous morbidity and mortality worldwide. Current estimates are that there are nearly 35 million people infected with HIV-1 globally, and that there are 15,000 new infections occurring daily (1). Developing countries shoulder nearly ninety five percent of all new HIV-1infections. Although recent advances have been made in antiretroviral therapy for HIV-1infection, there is no cure for AIDS, and the expense of drug therapy is prohibitive for most of the population. For this reason, clinical HIV-1 prevention trials sponsored by NIAID and the HPTN and HVTN are ongoing and in development. These trials include prevention through antiretroviral drugs, microbicides, behavioral approaches, barrier and other contraceptive/STD prevention methods, vaccines (in perinatal settings), chemoprophylaxis, treatment of sexually transmitted diseases, and combined approaches (2).

Since the primary end point of most prevention trial studies is incident HIV-1 infection, important immunologic and virologic observations can be made early after identification of infection. Current literature suggests that understanding events that occur early in HIV-1 infection can aid in predicting the subsequent course of disease, and therefore, perhaps impact treatment and care. For example, it has been shown that initial RNA viral load measurements are highly predictive of AIDS, and that subsequent serial CD4 and viral RNA measurements provided even better prognostic discrimination (3). It has also been hypothesized that the early interaction between viral replication and the immune response may influence a person's longitudinal pattern of immunologic and virologic parameters (4).

Another important virologic measurement that can be made in a large study of a diverse population of acute seroconvertors is the transmission of drug-resistant virus. Transmission of drug resistant virus has been well-documented (5;6). The availability of antiretroviral therapy to subjects enrolled in HPTN trials will vary greatly from site to site within the HPTN. Furthermore, sporadic dosage due to limited availability of drugs and patient non-compliance will be issues at sites where therapy is accessible. The effectiveness of long-term use of certain drugs and regimens in geographic areas with limited access to antiretroviral therapies can be addressed through samples collected at HPTU sites through this protocol. It is not well understood whether naturally occurring genetic mutations in drug naïve acute seroconvertors confer actual drug resistance *in vivo.* A study of drug naïve U.S. military personnel with recently acquired HIV-1infection revealed that up to 26% of those tested had genetic mutations in the RT and protease genes (7). Balotta et al reported similar findings of naturally occurring genetic mutations in drug naïve individuals in an Italian cohort of newly infected patients (8). These

studies, however, do not directly address the discrepancies between genotypic and phenotypic mutations in nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitor (PI) variants in recent seroconvertors, and whether these naturally occurring variants are relevant in the actual transmission of resistant virus. The HPTN, through specimens collected in this protocol, will have an opportunity to study the prevalence, geographic distribution and consequence of naturally occurring NRTI and PI-related mutations that occur in primary HIV-1 infection.

Immune responses to HIV-1 detected in acute infection are also thought to correlate with disease outcome. Loss of T cell function *in vitro* predicts progression to AIDS and a decrease in survival time (9). HIV-1-specific $CD4^+$ helper responses are known to play a key role in maintaining effective immunity in a subset of individuals, termed long term non-progressors (LTNP), who successfully control viral replication, and fail to progress to AIDS despite ten or more years of infection (10). Furthermore, Rosenburg et al showed that HIV-1 specific proliferative responses to p24 are inversely correlated with viral load (11). $CD4^+$ cells also most likely contribute to effective antiviral immunity by enhancing $CD8^+$ CTL activity, increasing production of antiviral cytokines and potentiating humoral immune responses (reviewed in (12;13).

There is now abundant evidence to suggest that a vigorous $CD8^+$ cytotoxic T lymphocyte (CTL) response, as well as a $CD4^+$ response, is critical for controlling HIV-1 replication and preventing progression to AIDS. The importance of virus-specific CTL in HIV-1 infection is illustrated by the observation that $CD8^+$ CTL responses are readily detectable early after acute infection and coincide with the resolution of high levels of initial viremia (14-17). Furthermore, LTNPs have been shown to maintain a high level of $CD8^+$ CTL activity to many epitopes of multiple antigens of HIV-1 and decline in this antiviral response coincides with disease progression (18-22). A vigorous HIV-1 specific $CD8^+$ CTL response following acute HIV-1 infection has been shown to be temporally associated with the control of viremia (23). Several investigators have shown that the detection of anti-gag CTL occurs early after seroconversion and that the level of anti-gag CTL response correlates with disease progression (24-26). Control of initial viremia is also crucial in the prevention of transmission of HIV-1 to sexual partners. Quinn and colleagues have recently shown that a strong correlation exists between viral load and the risk of transmission (27). A better understanding of the immunologic control of viral load will provide insights into modifying this important factor in HIV-1 transmission.

The observations described above have largely been made using samples from North American patients infected with Clade B isolates of HIV-1. The data on the cellular immune response to HIV-1 in other parts of the world to non-Clade B viruses is much more limited. There have been several studies on the CTL recognition to homologous and heterologous HIV-1 antigens from Clades E in Thailand (28) and Clades A, C and D in Africa (29;30). These studies have primarily been done for the purposes of addressing whether subtype-specific vaccines are necessary. Results from these studies have indicated that for more highly conserved proteins of HIV-1, such as gag, there is some cross clade recognition of several known, well-conserved gag epitopes (31-33). Conversely, in less conserved proteins, such as env and nef, cross clade recognition is not as consistent, especially in regions where known epitopes differ by one or more amino acids for a given allele (31-33). There remains, however, a clear gap in our knowledge of what represents a relevant HIV-1-specific immune response in non-Clade B

infections in other parts of the world. For example, a central question in HIV-1 vaccine development is how the high level of viral diversity within and across different clades of HIV-1 worldwide impacts CTL epitope recognition. A recent study by Goulder et al demonstrated two genetically distinct populations (North American Caucasians and South Africans) with different endemic viral subtypes had different patterns of immunodominance within the Gag region of HIV-1 (34). An expansion of the above studies within the genetically diverse populations of the HPTN will advance our knowledge of how the initial immune response impacts viral set point and ultimately impacts upon transmission and progression of disease.

1.2 Rationale

The rationale of this study is based on several premises:

- A primary endpoint of HPTN 034 is detection of recent HIV-1 infection.
- Investigations of the immunological, viral and host factors related to recent HIV-1 infection provide a unique opportunity to increase the understanding of factors impacting HIV-1 acquisition, HIV-1 transmission and subsequent HIV-1 disease progression, which is a stated goal of the HPTN.
- Most studies of primary HIV-1 infection have been limited to developed country settings and to individuals infected with HIV-1 subtype B infection.
- In addition to actively identifying individuals with recent HIV-1 infection as a primary endpoint for all of its clinical protocols, the HPTN 034 provides a unique opportunity to investigate the virological and immunological characteristics of recent HIV-1 transmission in settings with diverse host and viral factors.
- While protocols are under development to undertake HPTN clinical trials to prevent HIV-1 transmission in HIV-1 discordant couples (eg HPTN 052 and HPTN 039), a prospective observational study of recently infected individuals would provide an opportunity to establish the laboratory and clinical infrastructure to undertake these subsequent HPTN trials in a unique cohort of discordant couples, where the index partner is an individual with primary HIV-1 infection.
- In addition to their use in assays proposed in this protocol, reposited plasma/PBMC specimens from study participants with primary HIV-1 infection would provide a unique opportunity for future HPTN and collaborative laboratory studies.

The overall aim of this study is to investigate the immunologic, virologic and genetic factors that impact acute HIV-1 infection. Understanding how viral and host factors and the initial immune response HIV-1infection correlate to viral set point and disease progression represents a crucial aspect in designing prevention strategies involving the use of antiretrovirals, microbicides and vaccines.

An important question to be addressed in this protocol is whether a broadly cross-reactive and durable initial immune response to HIV-1 infection correlates with a lower viral set point and subsequent viral load trajectory in infected individuals across HPTN sites. A lower viral set point lowers the possibility of transmission. This will also potentially have implications in the design of subsequent vaccine and ART trials. Furthermore, understanding how secondary infections impact the body's ability to mount an immune response to HIV-1 infection or vice versa will advance our knowledge of how and when to treat these infections with the goal of ultimately reducing HIV-1 transmission.

The HPTN will encompass the largest network to date of cohorts of acutely infected individuals from countries around the world that continue to have a significant incident of HIV-1 infection. These cohorts will allow for the characterization of distinct viral subtypes and of the immune response to these different subtypes of HIV-1 in individuals with genetically diverse backgrounds (eg HLA, co-receptor genotype, etc), as well as a variety of factors that may impact the primary immune response to HIV-1 (eg. micronutrient deficiency, co-infections, viral load) Understanding the significance of the interaction of viral, genetic and immunologic characteristics in both individuals and populations and their relationship to mechanism(s) of transmission of HIV-1, is a primary goal of the HPTN. In addition, this HPTN 034A protocol would provide standardization of specimen collection for ongoing and future HPTN protocol specific-studies for the Pune site.

A secondary goal of this protocol is to begin to transfer the technology of virologic and immunologic assays to Pune, so that these measurements can be made on-site, instead of being shipped to and performed at a central location. Currently, novel immunologic assays, such as the ELISPOT and a flow cytometric assay for measuring intracellular cytokine levels are being developed and standardized for use on-site in large, multi-site clinical trials. The HPTN Core Laboratory will coordinate the development of consensus protocols and transfer of the technology for immunologic assays to Pune. This proposal will be coordinated with a companion 034 substudy (The HPTN HIV-Exposed Uninfected Study/HPTN 034B).

2.0 STUDY OBJECTIVES AND DESIGN

2.1 Primary Objectives:

- To compare initial HIV-1-specific CD8-mediated immune response with HIV-1 viral set point.
- To compare host immunologic genetic characteristics with HIV-1 viral set point, across diverse populations infected with different strains of HIV-1.

2.2 Secondary Objectives:

- To characterize the epitope specificity of the initial CD8-mediated immune response.
- To provide immunological and virological laboratory training and to establish laboratory quality control procedures for Pune.

- To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples.

- To establish a repository of plasma and PBMC specimens at the site in Pune from recently HIV-1-infected individuals, for future HPTN and collaborative immunologic and virologic studies

2.3 Study Design/ Participants:

This is a prospective cohort study to be conducted at NARI. The study will be performed in accordance with this protocol. Nari will document study data using standard data collection forms to be submitted to the HPTN Statistical and Data Management Center (SDMC).

It is anticipated that NARI will enroll an estimated 10-15 individuals/year with acute or recent HIV-1infectionAdditionally, blood samples from 20 low-risk seronegative volunteers (controls)/site will be collected at the beginning of the study, and then approximately 10/year.

Subjects from the 034 trial with documented recent HIV-1infection (i.e. HIV-1 antibody seroconversion, HIV-1 DNA or HIV-1 RNA PCR or p24Ag + in pre-antibody seroconversion "window" or detuned HIV-1 antibody EIA) within the past six months will be eligible for enrollment in this protocol.

Subject would be followed prospectively for 2 years. At each visit, a phlebotomy specimen and clinical data would be collected, according to the schedule provided in Table 1 (Appendix A). The laboratory assays proposed for each visit are also outlined in this table.

3.0 STUDY POPULATIONS

3.1 Inclusion Criteria Early HIV-1 Infection Subjects

One of the following must be fulfilled:

- Recent Infection: Documented HIV-1 antibody seroconversion within the past six months

OR

- Acute Infection: HIV-1 antibody negative or indeterminate and HIV-1 PCR + or p24 antigen positive

OR

- Recent Infection: As determined by detuned ELISA (This test is expected to be available within the next year. The reliability of this test is currently under evaluation(35))

All of the following must be fulfilled:

- Willing and able to provide informed consent
- Aged > 18 years
- Willing to permit phlebotomy of four tubes of blood (40 mls) every three months for 24 months

3.2 Exclusion Criteria

- An obvious psychological/psychiatric disorder that would invalidate the informed consent process or otherwise contraindicates participation in the study.
- Hemoglobin level < 7mgs/dl

3.3 Inclusion Criteria for HIV-uninfected Controls

- HIV-antibody negative using standard site specific screening algorithm
- Self-report at low risk for HIV-1 infection for prior one year and at the time of enrollment
- Willing and able to provide informed consent
- Aged > 18 years

3.4 Exclusion Criteria for HIV-uninfected Controls

- HIV-infected
- An obvious psychological/psychiatric disorder that would invalidate the informed consent process or otherwise contraindicates participation in the study
- Hemoglobin level < 7mgs/dl

3.5 Conditions for Participant Withdrawal

- A participant may be discontinued early from the study for any of the following reasons:
- Participant withdraws consent/decides that s/he does not want to continue in the study.
- A clinician or the site PI decides that continued participation is not warranted or advisable due to health reasons or other reasons related to the well-being of the participant, including an obvious psychological/psychiatric disorder that would invalidate the informed consent process, or otherwise contraindicate participation in the study.
- Any other condition that in the opinion of the study site Principal Investigator will interfere with achieving the study objectives. The decision to withdraw the participant should be done in consultation with the Protocol Co-Chairs and HPTU 034 Core Protocol Team.
- Reasons for discontinuation from the study will be recorded on the appropriate case report form.

4.0 STUDY PROCEDURES

This section refers to clinical and laboratory procedures that will be used to evaluate outcomes for each of the primary objectives. The procedures discussed below will be used to accomplish the primary and secondary objectives in this protocol.

4.1 HIV-1 Screening

HIV-1 screening will be performed as described in section 4.4.1

4.2 Enrollment

Enrollment will take place over the two years of the HPTN 034 protocol; participants will be followed for up to two years, if funding is available. The schedule for sample collection is outlined in Table 1. If it can be determined that participants were infected within the previous three months, samples would be drawn at enrollment, once a month for three months, and then every three months for up to two years. If participants have been infected for more than three months, and less then six months, specimens would be collected at enrollment, and every three

months for up to two years. Participants will be asked to provide a 40 ml blood sample (four EDTA vacutainer tubes, 10mls/tube), answer a short questionnaire and undergo a brief physical exam. PBMC and plasma will be stored on site for immunologic and virologic studies through the CL.

Study participants with recent HIV-1 infection will be also be strongly advised to refer their primary sexual partner(s) for HIV-1 screening and risk reduction counseling. Sexual partners of participants with recent HIV-1 infection who agree to HIV-1 screening will be determined to be either chronically HIV-1 infected of unknown duration, recently HIV-1 infected themselves, or HIV-1-uninfected. Those partners found to be recently HIV-1-infected will also be eligible for enrollment in this study. Partners found not to be HIV-1 infected will be eligible to enroll in a companion HPTN sub-study of exposed-uninfected partners (HPTN 034B).

Individuals with acute HIV-1 infection are highly infectious to their sexual contacts and with whom they may share needles. The ethical concerns surrounding this issue are important, complex and will require comprehensive review and discussion at each participating HPTU. Of particular importance are the ethical issues concerning contact tracing, protecting the confidentiality and human rights of the participants and their sexual contact, as well as access to the best available local care for HIV-1 infection. These issues are important for all HIV-1 prevention studies for which HIV-1 infection (i.e. HIV-1 antibody seroconversion) is the primary or secondary endpoint. However, they are particularly important in the setting of acute HIV-1 infection, due to the increased risk of secondary transmission. Participants enrolled in this study of acute HIV-1 infection will be strongly counseled to inform their sexual and needle sharing partners of their HIV-1 status. It is therefore critical that all participating HPTU sites are committed to providing the best available risk reduction counseling for study participants throughout their participation in this protocol.

Prior to enrollment, informed consent will be obtained and participants will be counseled appropriately about their HIV-1 status. Eligibility will be confirmed and locator information will be obtained. Demographic information will be obtained. A physical exam will be completed. If symptoms of a sexually transmitted disease are present at enrollment or during follow-up, diagnosis and referral for treatment will be provided as locally appropriate.

The schedule of visits and procedures is outlined in Table 1 (Appendix A). Specimens will be collected, processed and stored or shipped according to the protocols outlined in the site-specific study manual. The next appointment will be scheduled and the appropriate data collection forms will be completed.

At all visits, (enrollment and follow-up) whole anticoagulated blood will be obtained using four 10 ml lavender-top EDTA tubes. Blood should be processed within 12 hours of collection, as it has been shown that in HIV-infected individuals, the detection of cellular immune responses drops precipitously over time (J. Cox, personal communication). The NARI site laboratory will follow specific protocols for processing and storage of PBMC and plasma.

4.3 Follow-up

At each follow-up visit (seropositive subjects) the participant's identity and ID number will be verified. A physical exam and questionnaire will be completed. Specimens will be collected, processed and stored/shipped according the study-specific procedures manual and the schedule shown in Table 1 (Appendix A). The next appointment will be scheduled (if required) and the appropriate data collection forms will be completed.

To maximize efficiency, established systems for follow-up at NARI will be utilized to the extent possible, including the use of home visitors, modest incentives, and tracking procedures. If a participant misses a scheduled study visit, the study site staff will try to establish communication with the participant through all possible means using the contact information provided by the volunteer. The need to adhere to the visit schedule will be emphasized at each contact. Participants may be compensated for meals, time away from work and for transportation to the study clinic as appropriate.

4.4 Laboratory Assays

4.4.1 Diagnosis of HIV Infection:

As outlined in the 034 protocol we will utilize commercially available HIV antibody assay kits for HIV screening. To be documented as HIV infected for the purposes of this protocol, eligible subjects must have a current positive HIV antibody test and a negative HIV antibody test result within the prior 6 months. A positive antibody test would be defined as either A) HIV + by two different rapid tests B) HIV+ by two different serum ELISA tests C) HIV+ by one rapid AND one serum ELISA test or D) HIV+ by either a rapid test OR an ELISA AND by one HIV western blot. As outlined above, individuals who are NOT HIV antibody positive, based upon any of the above four criteria, may also be enrolled in this study if they are identified in the pre-antibody seroconversion "window".

4.4.2 HIV-1 Viral Load Testing:

The Pune HPTU site will be expected to perform HIV-1 PCR and viral load (VL) measurements for studies outlined in this protocol, as well as other potential HPTN protocols such as preparedness studies, perinatal studies, and ART protocols requiring VL measurements. The HCL will recommend the use of the Roche Amplicor HIV-1 Monitor™ Test version 1.5 because of its increased sensitivity and ability to detect non Clade B subtypes of HIV-1 (36). This assay would be recommended for sites interested in screening HIV-1 antibody negative specimens for identification of HIV-1 PCR+ subjects in the "window" period for enrollment in this protocol. This same assay would be utilized on-site for all VL assays for study participants. The HCL virology core will provide training for this HIV-1 PCR assay. This assay contains quality control material in the form of negative, low and high standards that will be used with each assay run. There is also an internal standard control that is assayed with each specimen. NARI will also be required to participate in QA proficiency testing on a quarterly or semi-annual basis through a

DAIDS-sponsored Virology Quality Assurance (VQA) program already in place in the US. More detailed information on the viral load assay is provided in Appendix C.

4.4.3 Specimen Processing and Storage

The HCL immunology group will provide training and technical support to the PUNE site in a number of laboratory techniques required for this protocol, including collection, processing and storage of patient specimens, as well as a the immunological assays required for this study. Initially, the HCL will focus on training and providing quality control assessments of the NARI HPTU in specimen collection and storage procedures. Proper specimen processing and storage is essential, prior to performing the immunological assays proposed for this study. This initial training can be performed using seronegative volunteer samples, to avoid using more limited seropositive samples. Therefore, for this purpose, NARI will be expected to enroll 20 seronegative volunteers at the on-set of the study, and then up to 10 seronegative volunteers for each year of the study.

The HIV-1 negative control samples will be used for:

- Quality controls for freeze/thaw and viability protocols
- HLA screening for population genetics
- Negative controls in future immunologic assays

The Pune HPTU must meet or exceed guidelines that will be outlined in the study-specific procedures manual for cell preparation and preservation quality (Appendix D). While it is clearly optimal to use freshly isolated PBMC for real-time assays, it is likely that in this study, and in future larger HIV-1 prevention and vaccine studies, the use of frozen specimens will be necessary for batch testing. It has been previously shown that cryopreserved PBMC can be used for detection of cellular immune responses in HIV-1-infected volunteers (28;37). The NARI site will be required to periodically perform quality control analysis of frozen cells by thawing single aliquots of cells from negative controls to assess cell number and viability (detailed in study-specific procedures manual). For example, it would be expected that NARI would be able to recover 50% or greater of the total number of PBMC stored, and that the viability of thawed cells would be approximately 90%. This number is based on previously observed recoveries from cryopreserved PBMC from HIV-1-infected individuals (38;39) Additionally, sites with flow cytometry capacities will be able to do more sophisticated measurements of cell preservation by testing for cell surface marker integrity. It has been previously shown that, cryopreservation of cells does not significantly alter the cell surface expression of most lymphocyte markers studied (40).

4.4.4 HLA typing

PBMC from HIV-1 uninfected individuals will also be used for HLA typing to be performed in collaboration with Dr. Thomas Williams' Laboratory at The University of New Mexico Health Science Center Albuquerque, New Mexico. NARI would be required to ship a single frozen vial of PBMC to this laboratory for HLA typing. There is increasing evidence that HLA type influences the course of a number of diseases such as HIV-1 and malaria (41;42). Furthermore, identification of CTL epitope specificities using HLA types for modeling binding motifs has

become a standard practice in cellular immunology. Therefore, characterizing the HLA types of the populations within the HPTN will be important in understanding risk of HIV-1 infection and the immune response subsequent to infection.

4.4.5 ELISPOT Assays

As mentioned above, NARI will be evaluated for their ability to collect and store blood specimens. Once NARI is able to adhere to the quality control requirements outlined above and in the site-specific study manual (Appendix D), they will be encouraged to participate training workshops at Johns Hopkins University as well as on-site and regionalized HPTN training. Training will be provided for participation in future virologic and immunologic studies of acute seroconvertors including measurements of T cell function through ELISPOT and flow cytometry analysis (where available). If procedural problems or inconsistencies occur in QC procedures, NARI will be responsible for internal problem solving, however, communication with the HCL group will be encouraged. Personnel from the HCL will periodically visit NARI for technical support and site evaluation.

NARI would be requested to identify at lease two key personnel on site as responsible for supervision and implementation of the study protocols. The site PI should be one of the site contacts, and the other should be a key laboratory scientist directly involved in the processing and laboratory testing of specimens. Representatives of NARI would be invited to join the Protocol Team to contribute to the design of the subsequent immunologic and virologic studies to be undertaken with the specimens in the site repositories. The HPTN Immunology Group will also invite and encourage participation and collaboration with investigators outside of the HPTN for future studies.

NARI will be trained to use the ELISPOT assay to measure the cellular immune response in individuals recently infected with HIV-1. This assay has been used previously to detect the $CD8^+$-mediated immune responses in HIV-1-infected individuals (34;43-47). The ELISPOT was selected for use at HPTU sites rather than the conventional CTL assay for several reasons. While the CTL assay is the "gold standard" for measuring $CD8^+$ function (*in vitro* cell killing) in HIV-1-infected individuals, it is extremely laborious and difficult to perform. It requires a long (14 day) *in vitro* stimulation period and the use of sodium ^{51}chromate. The CTL assay requires more than $20x10^6$ cells/assay, and is not considered to be a quantitative measurement of antigen-specific T cell frequencies. Recent studies have shown that the CTL assay and the ELISPOT assay are comparable in detection of $CD8^+$-mediated immune response in seropositive individuals (M. Altfeld, G. Ferrari, personal communications). Our laboratory as well as others has shown that the ELISPOT assay can be performed using cryopreserved PBMC. The ELISPOT assay is fairly simple to perform and requires only a brief, overnight *in vitro* stimulation period. It requires a fraction of the cells necessary for a comparable CTL assay. For a more comprehensive discussion of cellular assays currently in use for clinical immunology assessments, please refer to current reviews by Hickling and Whiteside (47;48).

The ELISPOT assay is an alternative functional assay that can measure $CD8^+$ T cell function. The read-out of the ELISPOT assay is secretion of IFN-γ from HIV-1-specific T cells. Secreted IFN-γ is captured onto a membrane-coated ELISA plate by an IFN-γ-specific monoclonal antibody. It is then detected using a second biotinylated IFN-γ-specific monoclonal antibody, and

developed using standard ELISA reagents. The number of spots counted in a particular sample well corresponds to the number of HIV-1-specific T cells in the well/total number of cells added. Therefore, the relative frequency of cells that are specific for a particular antigen can be determined. This assay can also measure a positive and negative response for each sample tested. The positive control consists of the addition of PHA to replicates of PBMC samples. A majority of $CD3^+$ lymphocytes will secrete IFN-γ in response to this mitogen. The negative controls for this assay are mock-stimulated replicates in which no stimulus is added. This allows for the assessment of background levels of IFN-γ secretion. For purposes of quality assurance, the HCL will create a panel of frozen PBMC to serve as positive and negative control samples. These cells will be distributed to each participating HPTU site to test in a blinded fashion in the ELISPOT assay using a standard set of reagents.

An important issue for the ELISPOT assay is the choice of HIV-1-antigens to use for the *in vitro* stimulation. The use of overlapping pools of HIV-1 gag peptides for *in vitro* stimulation has been shown to be an effective means of characterizing the gag-specific immune response to HIV-1 in acute and chronic infection (34;43-47). One complicating factor that will need to be addressed is whether to use sequence-specific HIV-1 antigens for the *in vitro* stimulation. Designing peptides that match the predominant transmitting strain of HIV-1 in the transmitting population in Pune would be extremely difficult and expensive. Furthermore, because there is limited data on the importance of customizing overlapping peptide sequences to match subtypes of HIV-1 represented at each site, it is not clear that this would outweigh the substantial cost of synthesizing multiple sets of peptides. Therefore, the HCL will attempt to provide reagents that are based on the best information currently available on viral subtype amino acid sequences. These sequences will be obtained from sequencing data from the sites themselves, sequences published in the current literature, and the compendium of sequence data from the HIV-1 molecular database at the Los Alamos National Laboratory (49).

Based upon the data from prior studies and an assessment of the logistical limitations of initiating this study, this proposal will initially focus on measurement of HIV-1 gag-specific ELISPOT responses utilizing a matrix format of pools of overlapping HIV-1 gag peptides (24-26;50). NARI will initially be provided with a set of HIV-1 subtype C gag peptides. Based upon this initial analysis of the gag-specific responses and upon data from other ongoing studies, we propose to consider expanding this analysis to include assessment of other HIV-1 antigens, including env, pol, nef and tat. The details of the proposed consensus ELISPOT assay with gag peptides are provided in Appendix D.

ELISPOT measurements will be made as early and frequently as possible after initial identification of infection. The visit schedule is outlined in Appendix D, and described in detail in Section 2.3 of this protocol. The need for these frequent blood draws is comes from data that suggests that the epitope recognition seen during the acute phase of infection differs substantially from the immune response seen in chronic phase (B. Walker, M. Altfeld, personal communication). Therefore, it will be crucial to make these early measurements of viral load and HIV-1-specific immune responses, whenever possible.

Based upon the analysis of the ELISPOT data, a decision will be made by a consensus of the protocol team, which includes NARI site investigators and investigators outside of the HPTN,

about expanding the analyses to other immunological assays (e.g. tetramer, intracellular flow cytometric assays, co-receptor genetic analysis), other antigens (e.g. nef, pol, env, tat) and other critical scientific questions. New information is continuously being generated that impacts our understanding of events critical to HIV-1 transmission and pathogenesis. To optimize the relevance of this study to the HPTN, the protocol team must include outside expertise and must stay informed about the latest information that might suggest modifications in the specifics of the laboratory assays utilized for this protocol. This protocol must also facilitate and be coordinated with ongoing, related laboratory studies at participating HPTUs, as well as with outside collaborators. The science in this field moves quickly and this laboratory protocol must remain dynamic, flexible, informed and collaborative to optimize this important scientific opportunity.

5.0 EVALUATION OF OUTCOMES

5.1 Primary Outcomes

Following the successful implementation of immunologic (ELISPOT) and virologic (viral load measurements) assays at NARI (See quality control and training details above), this project will collect data to address the two stated primary objectives.

5.1.1. Primary Objective (a).

To compare initial CD8-mediated immune response with HIV-1 viral set point

The initial research hypothesis for this objective, based upon prior US-based studies of individuals infected with HIV-1 subtype B, is that HIV-1-infected individuals with early $CD8^+$-mediated recognition of multiple HIV-1-gag epitopes (polyclonal response) will demonstrate a lower HIV-1 viral set point, compared with individuals that do not recognize multiple HIV-1 gag epitopes. A further hypothesis is that this correlation will be demonstrated in recently infected individuals across diverse populations infected with different strains of HIV-1. The primary outcome measures for this analysis will be HIV-1 viral set point, defined as the median viral load between 6 and 12 months after estimated time of primary infection, and quantification of the number of different HIV-1 gag peptides recognized by each individual, as measured in an ELISPOT assay. In cases where positive responses are detected in the initial screen of the ELISPOT assay, additional assays will be performed on stored specimens to determine the phenotype of the responding cell, epitope specificity and MHC restriction. Based upon this initial analysis of gag-responses and upon data from other ongoing studies, we propose to consider expanding this ELISPOT analysis to include assessment of recognition of other HIV-1 antigens, including env, nef and tat.

5.1.2 Primary Objective (b).

To compare host immunologic genetic characteristics with HIV-1 viral set point.

The initial research hypothesis for this objective, based upon prior studies in a few countries demonstrating that certain HLA class I alleles may be associated with a slower disease HIV-1 progression, is that there are specific host genetic characteristics that will be associated with a lower HIV-1 viral set point in individuals from non-North American populations

infected with different strains of HIV-1. The primary outcome measure for this analysis will be HIV-1 viral set point, defined above, and HLA class I allelic genotype. Based upon this initial analysis of the HIV-1 class I alleles and upon data from other ongoing studies, we propose to consider expanding this analysis to include assessment of HLA Class II alleles and co-receptor genotyping.

5.1.3 Primary Endpoints

(a) Median HIV-1 viral load between 6 and 12 months after estimated time of primary infection
(b) ELISPOT measurement of HIV-1 peptide-specific T lymphocytes
(c) HLA-Class I MHC alleles

5.2 Secondary Outcomes

This protocol will also address a number of secondary objectives.

5.2.1 Secondary Objectives (a)

To characterize the epitope specificity of the initial CD8-mediated immune response.

For subjects demonstrating a response to HIV-1-gag peptides, the NARI site will be provided an opportunity to further characterize the HLA-restriction and minimal peptide epitopes of these responses.

5.2.2 Secondary Objective (b)

To provide immunological and virological laboratory training and to establish laboratory quality control procedures for the NARI HPTU.

Assessment of on-site capability to perform HIV-1 viral load and ELISPOT analyses will include participation in the quality control procedures outlined above.

5.2.3 Secondary Objective (c)

To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples.

This laboratory study will provide an opportunity to assess the feasibility of establishing and following cohorts of recently infected individuals and their sexual partners for future HPTN trials. Therefore enrollment and retention rates will be enumerated and evaluated.

5.2.4 Secondary Objective (d)

To establish a repository of plasma and PBMC specimens from recently HIV-1-infected individuals, for future HPTN and collaborative immunologic and virologic studies.

Remaining plasma and PBMC specimens from study participants will be reposited on-site and provide opportunities for HPTN and non-HPTN investigators to propose additional site specific and collaborative laboratory studies in the future.

5.2.5 Secondary Endpoints:

(a). Characterization of MHC Class I restriction and minimal HIV-1 peptide sequence of viral-specific T lymphocytes in early HIV-1 infection.

Quality control assessment of HIV-1 viral load and ELISPOT assays at the Pune site.

(b). Measurement of enrollment and retention of study participants (and their primary partners, if enrolled in the HPTN 034B Exposed-Uninfected Sub-study)

6.0. DATA COLLECTION

6.1 Data Forms

Data will be collected on standardized forms to be outlined in the site-specific procedures manual. An appropriate system for data transfer from the sites to the HCL and for central data management, quality assurance and analysis will be developed and utilized. Data and care report forms flow chart will be included in the study specific procedures manual along with procedures for data collection, transfer, and management.

6.2 Record Storage and Archive

The study site principal investigator and laboratory personnel will maintain all source documents used to complete case report forms including laboratory requisitions and reports, documentation of referrals, and progress notes. Specimens will be tracked using the Laboratory Data Management System (LDMS). All data collection forms and source records must be kept in locked files in a secure area. All study documents with participant identifying information including locator information and informed consent forms must be kept in locked files in a secure area apart from all other study documents. Study records must be available at all times for review by FHI, the study sponsor (NIAID) or their agents.

6.3 Statistical Considerations and Sample Size Calculations

Based upon assumptions outlined below for each of the two primary objectives, the target samples size for this study is a minimum of 20 subjects with recent HIV-1-infection retained for at least 1 year of follow-up. For both primary objectives, HIV-1 viral set point will be an outcome measure. Subjects will be stratified according to median viral load measured between 6 and 12 months after estimated time of primary infection (See Table 1. viral set point=median VL

of visits 5-8). Based upon this calculation, subjects would be stratified into the following categories: <10000 copies/ml, 10000-75000 copies/ml, >75000 copies/ml.

6.3.1 Primary Objective (a).

Gag-specific ELISPOT Response vs. HIV-1 Viral Set Point.

For the purposes of comparing ELISPOT response to viral load set point, gag-specific ELISPOT responses will be stratified into two categories: Responses recognizing two or fewer HIV-1 gag peptides and those responses recognizing more than two different HIV-1 gag peptides. As outlined in Appendix E, all experiments will be performed in duplicate wells. The IFN-γ-expressing spots will be calculated as the number of spots per 100,000 stimulated PBMCs. Results will be expressed as net peptide-specific IFN-γ spots / 10^5 PBMC. A positive peptide-specific response (responders) will be defined as a mean of greater than 5 spots above the mean spots of the mock-stimulated PBMC. Study participants will be expected to have multiple ELISPOT assays within the first 12 months of follow-up (See Appendix A).

This is a descriptive sub-study proposed for HPTN 034. Therefore, it is not expected that we will have sufficient sample size to definitively address the primary endpoint. Never the less, we will undertake the analysis as outlined on our projected sample size of 20-30 individuals. However, we have also projected that data from this sub-study would also be available for combining with similar data from other HPTN protocols in the future. Our subsequent "meta-analysis" will compare HIV-1 viral set point with the recognition of more than two (>2) gag peptides at any time point within 12 months since estimated time of infection. Separate and similar analyses will be performed for recognition of HIV-1 subtype B gag and non-B gag peptides. Additional analyses will determine if other ELISPOT outcome measures, such as consistent recognition of more than 2 gag peptides at multiple time points, also correlate with viral set point. We predict a 30% difference between the groups with respect to the proportion of individuals who elicit a broad immunologic response (defined above). With a sample size of n=39 per arm from multiple HPTN protocols in the future, we will have 80% power to detect a 30% difference (p=0.05).

Sample Size Estimate

Percent ELISPOT response to >2 HIV-1 gag peptides			Estimated Sample Size	
<10000 RNA copies/ml	10000-75000 RNA copies/ml	>75000 RNA copies/ml	N per arm	Total N
70%	50%	30%	93	279
80%	**50%**	**20%**	**39**	**117**
90%	50%	10%	20	60

6.3.2 Primary Objective (b).

HLA Class I alleles vs. HIV-1 Viral Set Point.

A similar "meta analysis" of data from 034A and other similar sub-studies is anticipated for this objective. The proportion of individuals with specific HLA Class I alleles will be compared with the viral load set point. Using HLA A2 as an example (known to be associated with a frequently measurable immune response to gag peptides), we posit that a higher percentage of individuals with this allele will fall into the low viral set point category. Again, we will have an 80% power to detect a 30% difference in the proportion of individuals with HLA A2 given a sample size of 39 individuals per arm.

7.0 HUMAN SUBJECTS CONSIDERATIONS

7.1 IRB Review

This protocol and the proposed informed consent documents and any subsequent modifications will require review and approval by a number of Institutional Review Boards or Ethics Committees, responsible for oversight of the HPTN activities. The activities of this protocol will be covered by an approved HPTN OPRR International Cooperative Project Assurance (ICPA) for each participating site, required for participation in an OPRR-recognized HPTN Cooperative Protocol Research Program (CPRP). It will be the responsibility of participating site investigators to obtain and document all required approvals for this study. Specifically site participation in this protocol will require documentation of ethical review and approval from the following:

NARI's local institutional IRB and Johns Hopkins JCCI

7.2 Informed Consent

Participation in this study will require a two-step informed consent process. A separate informed consent will be required for initial screening for HIV-1 infection (from the HPTN 034 study). HPTN 034 has proposed HIV screening and cohort enrollment consent procedures outlined in the full protocol. Based on eligibility criteria outlined in the protocol above (Section 3.1), a separate consent will be required for enrollment and participation in this prospective study of recent HIV-1 infection. The informed consent form and consent process will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. The consent form and study information will be provided in locally appropriate languages. A copy of the signed consent form will be given to the subject. NARI will be required to obtain review and approval of the local language consent form for their sites. Sample sub-study consent forms (HIV-infected and HIV-uninfected control forms) are provided in Appendix II of the full protocol, which include the specific information that should be communicated to each potential participant about the risks and benefits of participation in this study.

7.3 Confidentiality

All local study records will be kept in locked file cabinets in areas with limited access; databases will be secured with password-protected access systems. A coded ID number will identify all study data and specimens only, and records containing participant names or other personal identifiers, such as locator forms and informed consent documents will be stored separately from coded ID records.

7.4 Benefits

Support for HIV-1 Prevention

During the study, the participant will receive the most current information and counseling about how to avoid acquiring or transmitting HIV-1 to partners. Studying immune responses in early HIV-1 infection will provide important knowledge concerning strategies for prevention and vaccine development and evaluation. In light of the high risk of secondary HIV-1 transmission to sexual partners from individuals with recent HIV-1 infection, an important effort will be made to encourage participants to refer their partners for HIV-1 screening and counseling.

Access to Care

This protocol will require documentation from each participating site that the current UNAIDS/CIOMS guidelines for provision of care and human rights protection is provided for all HIV-infected and HIV-uninfected participants. Documentation that diagnosis and treatment meets or exceeds the best available clinical care for their own local situation will be required. This includes diagnosis and treatment of sexually transmitted diseases, as well as the complications of HIV-1 infection. In addition, documentation will be required that the local IRB has specifically reviewed and addressed this important issue, as part of their ongoing review of this protocol. NARI will provide current local guidelines for clinical care of HIV-infected patients available in-country and provide mechanisms for appropriate treatment referrals to all participants regardless of stage of disease. NARI will be responsible for assuring compliance with their own local ethical guidelines for providing clinical care to study participants. Since primary HIV-infection is a clearly defined outcome measure for all HPTN protocols, it is expected that the HPTN and/or DAIDS will develop guidelines for the care and support of international and domestic HIV-infected participants for all HPTN protocols, including this laboratory protocol. This protocol would be expected to follow all such HPTN/DAIDS guidelines, at that time. Additional details addressing this important issue are provided in the full HPTN 034 protocol.

7.5 Risks

Health Risks
Study participants may feel discomfort associated with phlebotomy, including pain, bruising and/or in very rare instances, local infection.

Psychosocial Risks
Both HIV-1 infected and uninfected volunteers will be recruited for the study, therefore there is no risk of being labeled as infected as a result of participation in the study. Periodic testing of viral and immunologic indices may increase participant's anxiety level concerning their health.

7.6 Study Discontinuation

The study may be discontinued at anytime by FHI, the HPTN, NIAID, by the Indian government, the ICMR or NARI.

7.7 Incentives

Provided approval is obtained from the local IRB/EC, participants may be provided with a small incentive in the form of a transport allowance, lost work, child care and free access to medical services for their participation in this study, as determined by individual sites based on local standards of practice and in accordance with ongoing studies from which the participants will be identified. The details of these incentives will be included in the local IRB approved consent form.

8.0 LABORATORY SPECIMENS AND BIOHAZARD CONTAINMENT

As the transmission of HIV-1 and other blood-borne pathogens can occur through contact with contaminated needles, blood, and blood products, appropriate precautions to handle blood and bodily secretion will be employed by all personnel when drawing of blood and shipping and handling of all specimens for this study, as currently recommended by the Centers for Disease Control and Prevention.

9.0 ADMINISTRATIVE PROCEDURES

9.1 Study Coordination

Close cooperation between the protocols team and site investigators, protocol coordinator, data managers and biostatisticians will be necessary; study progress will be evaluated by the study team on a regular basis via conference calls. The sites will employ a common study specific procedures manual (with site-specific sections as appropriate). This manual will outline procedures for enrollment, follow-up, data and forms processing and other study operations.

A common study laboratory manual will be followed to standardize specimen collection, preparation, testing, processing, and shipping. For laboratory procedures and assays (i.e., cell separations, freezing of samples, flow cytometry, etc.) that can be performed by the local site laboratory, or the CL. Other assays will be performed at the HCL or in the laboratories of non-HPTN participants/collaborators.

Every effort will be made to provide training and technology transfer to each of the sites to increase their overall capabilities. There will also be a strong commitment to rapid bi-directional exchange of test results and other data to facilitate the collaborative interpretation and timely publication of HPTN findings.

9.2 Study Monitoring

The HCL will conduct site visits to monitor adherence to human subjects and other applicable regulations, adherence to the study protocol and procedure manuals, and the quality of the data collected at the site in Pune. The study site key investigators will allow the inspection of study documents (e.g., consent forms, process data collection forms, questionnaires) for confirmation of the study data.

9.3 Protocol Compliance

This study will be conducted in full compliance with the protocol. The protocol will not be amended without prior written approval by the Protocol Chairs and DAIDS Medical Officer. Protocol amendments requiring IRB approval must be approved by the IRB prior to implementing any amended procedures.

9.4 Investigator Records

All records must be retained on-site throughout the study's period of performance. The HPTN will provide each site with written instructions for long-term record storage at the completion of the period of performance.

Study records include the following:
Administrative files, including initiation documents and all reports and correspondence relating to the study.
Records for each participant, including informed consent forms, locator forms, data collection forms, and source documents

9.5 Use of Information and Publications

The policies that govern specimen ownership, intellectual property rights, and the publication/authorship of research will be derived from the WHO policy document concerning these issues. Viral isolates and any remaining serum/plasma/cell samples, which are not consumed during the specified testing, will be retained in a specimen archive at the local site, the HPTN laboratory, or the NIAID Repository contractor (BRInc.) in Bethesda Maryland. These samples will be available to any responsible investigator for further studies, pending review of the request by the site that submitted the sample and the HPTN Steering Group or the appropriate oversight committee following HPTN. Publication of the results of this study will be governed by policies of the ICMR, DAIDS and the HPTN. Any presentation, abstract, or manuscript will be made available by the investigators to DAIDS and the HPTN manuscript review committee for review and comment prior to submission.

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APPENDIX A

Table 1. Scheduled visits for acute seroconvertors

	Screening	Enrollment														
Month	Pre	0	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Study Visit	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
Patient ID	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Physical Exam	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Questionnaire	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Assays:																
HIV-1 Test[1]	X															
HIV-1 RNA PCR (VL)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Storage:																
Plasma		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PBMC		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HLA Typing		X														
Total blood volume (ml)	10	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Number of tubes [2]	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

[1]Assay (PCR, ELISA, detuned assay will be site-specific)
[2]Tubes will be 10ml lavender top tubes containing EDTA.

APPENDIX B:

Table 2. Scheduled visits for seronegative volunteers.

	Screening	Study visit
Month	0	6
Study Visit	01	02
Patient ID	X	X
Questionnaire	X	X
HIV-1 Test[1]	X	X
PBMC (freezing)		X
HLA Typing		X
Total blood volume (ml)	5	20
Number of tubes drawn[2]	1	2

[1]Assay (PCR, ELISA will be site-specific)
[2]Tubes will be 10ml tubes lavender top tubes.

Appendix C: HIV Viral Load Measurement

Procedure:
AMPLICOR HIV-1 MONITOR TEST Version 1.5—STANDARD METHOD

SPECIMEN:

Plasma

Type: Blood will be collected in EDTA tubes.

Serum specimens and specimens collected in **heparin** are unsuitable for this test.

Standard precautions will be observed for the collection, handling, transport, and processing of all specimens.

Handling Conditions:

Follow procedures outlined in the PBMC isolation protocol.

MATERIALS AND EQUIPMENT:

Materials:

AMPLICOR HIV-1 MONITOR test kit, v 1.5

(1.) Specimen Preparation Reagents
AMPLICOR HIV-1 MONITOR Lysis Reagent
AMPLICOR HIV-1 MONITOR Quantitation Standard
AMPLICOR HIV-1 MONITOR Specimen Diluent
Isopropanol
70% Ethanol (**not denatured**), v/v with deionized water

(2.) Control Reagents
Negative Plasma (NHP)
AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)
AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)
AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)
VQA Controls (For ACTG samples/studies)

(3.) Amplification Reagents
AMPLICOR HIV-1 MONITOR Master Mix
AMPLICOR HIV-1 MONITOR Manganese Solution

(4.) Detection Reagents
MONITOR Denaturation Solution
MONITOR Hybridization Buffer
AMPLICOR Avidin-HRP Conjugate
AMPLICOR Substrate A
AMPLICOR Substrate B
AMPLICOR Stop Reagent
AMPLICOR 10X Wash Concentrate
Distilled or deionized water
AMPLICOR HIV-1 MONITOR Microwell Plate

Reagents not included in kit

(1) 95% ethanol (freshly diluted to 70% with deionized water)

(2) Isopropanol, reagent grade

Equipment:

(1.) Perkin Elmer GeneAmp® PCR system 9600 or 2400 thermal cycler

(2.) Consumables: tubes (MicroAmp Reaction Tubes PE #N801-0533),
Caps (PEN801-0535)
Base(PE N801-0531)
Tray and retainer (PE N801-0536)

(3.) Aerosol resistant pipette tips capable of holding 50 to 1000 microliters to prevent specimen and amplicon contamination

(4.) Eppendorf repeater pipet and 1.25ml Combitip Reservoir

(5.) Pipettors, adjustable volume (20 – 200 µl), (50 µl), (200 µl) Pipettes should be within 3% of stated volume.

(6.) Impact Pipettor

(7.) Narrow tip, sterile transfer pipettes

(8.) Latex or nitrile gloves, **powder-free**

(9.) Cryovials with caps, sterile, 2ml capacity. Screw caps must be used to prevent specimen splashing and potential contamination No snap caps

(10.) Tube racks for cryovials

(11.) Microwell plate sealers

(12.) Disposable reagent reservoirs

(13.) Disposable plastic bags

(14.) Biological Safety Cabinet (BSC), Template Tamer Box, or equivalent

(15.) Microplate Washer capable of washing a 96-well plate with 350-450 ul at 30 second intervals

(16.) Microplate Reader with the following specifications: bandwidth = 10 $\pm$ 3 nm, absorbance range
= 0 to a minimum of 2.00 when read at 450 angstroms, repeatability = 1% accuracy
= 3% from 0 to 2.00 when read at 450 angstroms, drift <0.01 per hour

(17.) Centrifuge

(18.) Microcentrifuge max RCF 16,000 x g, min RCF 12,500 x g

(19.) Vortex mixer

(20.) Dry incubator 37°C($\pm$ 2°C)

(21.) Personal computer with spreadsheet software

(22.) Graduated cylinders, 100 to 1000 microliter capacities

(23.) Sterile bottles

(22.) Disposable pipettes, 5ml and 10ml

(23.) Absorbent backed paper

(24.) Disposable gown

(25.) Clorox or equivalent

WARNINGS AND PRECAUTIONS:

(1.) Do not pool reagents from different lots or from different bottles of the same lot.

(2.) Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

(3.) Do not use kit after expiration date.

(4.) Material Safety Data Sheets (MSDS) are available on request from Roche Response Center or your local Roche office.

(5.) Three work areas are required: 1) a Rnase free area, 2) a Pre-Amplification extraction area and 3) a Post Amplification area. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification Area. Pre-Amplification activities must begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Gloves must be worn in each area and removed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-Amplification supplies and equipment must be confined to the Post-Amplification Area at all times.

(6.) This kit contains a component (NHP) derived from human blood. The source material has been assayed by the US FDA approved tests and found non-reactive for the presence of Hepatitis B Surface Antigen and antibodies to HIV-1-1/2 and HCV. No known test methods can offer complete assurance that product derived from human blood will not transmit infectious agents. Therefore, NHP should be handled as if infectious.

(7.) HIV-1 QS, HIV-1 DIL, HIV-1 MMX, HIV-1 Mn^{2+}, HIV-1 low, and high positive control contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing materials down laboratory sinks, flush the drains with large amounts of water to prevent azide buildup.

(8.) Wear eye protection, laboratory coats and disposable gloves when handling HIV-1 LYS, HIV-1 MMX, Monitor Denaturation, Monitor HYB, AV-HRP, SUB A, SUB B, Working substrate mixed, and Stop solution. Avoid contact of these materials with the skin, eyes, or mucus membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

(9.) SUB B and Working Substrate contain dimethylformamide, which has been reported to be toxic in high oral doses and may be harmful to the unborn child. Skin contact, inhalation of fumes and ingestion should be avoided. If skin contact occurs, wash thoroughly with soap and water and seek medical advice immediately.

(10.) Do not allow HIV-1 LYS, which contains guanidine thiocynate, to contact bleach. This mixture can produce a highly toxic gas.

(11.) Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. Do not use snap cap tubes.

PREPARATION:

Specimen Preparation Reagents:

(1.) AMPLICOR HIV-1 MONITOR Lysis Reagent

A tris buffered solution containing guanidine thiocyanate, dithiothreitol and glycogen.

Store at 2 to 8°C until expiration date. Warm at 30-37°C to dissolve any salt crystals that form.

(2.) AMPLICOR HIV-1 MONITOR Quantitation Standard (QS)

A buffered solution containing Quantitation standard RNA, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(3.) Working Lysis Reagent

Add 100µl of the QS to one bottle of Lysis reagent. Mix thoroughly. The pink dye is used as a visual confirmation that the QS has been added to the lysis reagent. Discard remaining QS. Store at room temperature and use within 4 hours.

(4.) AMPLICOR HIV-1 MONITOR Specimen Diluent

A buffered solution containing Tris-HCl, EDTA, poly rA RNA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(5.) Isopropanol(2,2 – proponal)

Store at room temperature in the flammable liquids cabinet.

(6.) 70% Ethanol (not denatured), v/v with deionized water

Dilute absolute ethyl alcohol with distilled, deionized water to a 70%. (Volume depends on the grade of ethanol – 95% to 100%). Store at ethanol in flammable liquids cabinet. Prepare fresh 70% daily.

Control Reagents:

(1.) Kit Controls

A. Negative Plasma (NHP)

Human plasma that has been found to be non-reactive by FDA licensed test for antibody to HCV, antibody to HIV-1/2, and HbsAg, containing ProClin 300. Store at 2 to 8°C until expiration date.

B. AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)

A buffered solution containing poly rA RNA, Tris-HCL, EDTA, and 0.05% sodium azide.

Store at 2 to 8°C until expiration date.

C. AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)

A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide.

Store at 2 to 8°C until expiration date.

D. AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)

A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide.

Store at 2 to 8°C until expiration date.

Amplification Reagents:

(1.) AMPLICOR HIV-1 MONITOR Master Mix

A bicine buffered solution containing <37% glycerol, potassium acetate, <0.07% dATP, dCTP, TTP, dUTP, biotinylated primers, <0.0004%r Tth Pol, <0.01% AmpErase and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(2.) AMPLICOR HIV-1 MONITOR Manganese Solution

A solution containing manganese acetate and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(3.) Working Master Mix

Add 100µl of AMPLICOR HIV-1 MONITOR Manganese solution to one tube of AMPLICOR HIV-1 MONITOR Master Mix. It is not necessary to measure the volume of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10 – 15 times or by mixing with a vortex for 3 – 5 seconds.
The pink dye is used for visual confirmation that the Manganese solution has been added. Discard the remaining Manganese Solution. Working Master mix should be stored at 2 - 8°C and use within 4 hours.

Detection Reagents:

(1.) MONITOR Denaturation Solution

A solution of EDTA, 1.6% sodium hydroxide, and amaranth dye. Store at 2 to 25°C until expiration date.

(2.) MONITOR Hybridization Buffer

A sodium phosphate solution containing <0.2% solubilizer and <25% sodium thiocyanate. Store at 2 to 25°C until expiration date.

(3.) AMPLICOR Avidin-HRP Conjugate

An avidin-horseradish peroxidase conjugate in a buffered solution containing 1% ProClin 150™, emulsifier, bovine gamma globulin and 0.1% phenol. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first.

(4.) AMPLICOR Substrate A

A citrate solution containing 0.01% H_2O_2 and 0.1% ProClin 150. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first.

Do not expose to metals, oxidizing agents or direct light.

(5.) AMPLICOR Substrate B

Contains 0.1% 3,3',5,5'-tetramethylbenzidine in 40% dimethylformamide. May cause harm to an unborn child. Harmful by inhalation and contact with skin. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first. Do not expose to metals, oxidizing agents or direct light.

(6.) Working Substrate Solution

For each microplate, mix 12ml of Substrate A with 3ml of Substrate B. Protect from light. Store at room temperature. Use within 3 hours. Do not expose to metals, oxidizing agents or direct light.

(7.) AMPLICOR Stop Reagent

Contains 4.9% sulfuric acid. Store at 2 to 25°C until expiration date.

(8.) AMPLICOR 10X Wash Concentrate

A sodium phosphate and sodium salt solution containing EDTA, <2% detergent and 0.5% ProClin 300. Store at 2 to 25°C until expiration date.

(9.) Wash Buffer 1X

Dissolve any precipitate by warming to room temperature. Add 100ml of 10X wash concentrate to 900ml of distilled, deionized water. Mix well. Store in a clean, closed container at 2 to 25°C for up to 2 weeks. Label with preparation and expiration dates.

(10.) AMPLICOR HIV-1 MONITOR Microwell Plate

An oligonulceotide probe coated microwell plate with twelve 8 well strips in one resealable pouch with desiccant, HIV-specific DNA probe (rows A-F), Quantitation Standard specific DNA probe (rows G-H).
Store at 2 to 8°C in the foil pouch. The plate is stable in an unopened pouch until the expiration date. Once opened the plate is stable for 3 months or until expiration date, whichever comes first) as long as it is store in the resealable pouch.

CALIBRATION:

None

QUALITY CONTROL:

It is recommended that one replicate of each of the following controls: HIV-1 Monitor (-), (L+), and (H+) be included in each test run. As with any new laboratory procedure, new operators should consider the use of additional controls until such a time as high degree of confidence is reached in their ability to perform the test correctly.

All controls and patient specimens should yield OD values for the QS that meet the criteria described in the Results section, demonstrating that the specimen processing, reverse transcription, amplification, and detection steps were performed correctly. If any specimen has a QS OD value that does not meet the criteria described above, the result for that specimen is invalid, but the run is still acceptable. If any control has a QS OD value that does not meet the criteria described above, the entire run is invalid.

The expected range for each of the controls is specified for each lot of control and is provided with the kit. The copy number/ml should fall within the range indicated on the provided sheet. This range is to be used until at least 10 values have been run and an in-laboratory range can be established. These ranges should be checked periodically and any trends notes. The negative control should yield a "not detected" result; i.e. all HIV OD values less than 0.200. If one of the positive control values falls outside of the 2 standard deviations, but within 3 standard deviations, the run can be accepted. When the assay is run the next time and same control is out, further investigation needs to be done. If both positive

controls have values outside of 2 standard deviations but within 3 standard deviations, the results should be reviewed by the laboratory director for final approval. If one of the positive controls falls outside of 3 standard deviations while the other positive control is in, the results need to be reviewed, with the possibility of the range of values around the out of control to be repeated. If the next time the assay is run and the same control is out of range then further investigation is needed.

It is required that the results be reviewed by the supervisor, director, or designee before they are released.

TROUBLESHOOTING/MAINTENANCE:

Maintenance is to be performed on the plate washer, reader, and thermal cycler. The maintenance on the plate washes will vary depending on the model and brand but the system should be bleached at least monthly. The plate reader needs to be calibrated monthly. There is daily, weekly, monthly and semi- annual maintenance to be performed on the thermal cycler. Check the Perkin Elmer manual.

PROCEDURE-STEPWISE:

Preliminary Statements

All reagents should be at room temperature before using them.

Workflow in the laboratory should proceed in a uni-directional manner, beginning in the Reagent Preparation Area and moving to the Specimen Preparation Area and then to the Amplification/Detection Area.

Run Size (hospital patients): Each kit contains sufficient reagents for 2 -12 test batches, which may be performed separately or simultaneously. It is recommended that one replicate of the HIV-1 Monitor (-) control, (L+) control, and (H+) control be included in each run.

Workflow: The AMPLICOR HIV-1 MONITOR Test can be completed in one day or over two days. If the testing is to be done in a single day, follow the instructions in order. If the testing is to be completed in 2 days the procedure may be stopped after specimen preparation or after amplification. To perform the specimen processing on day one and amplification/detection on day two complete section B through step where you will freeze the samples at -20°C until amplification can occur. On day 2 begin with section A, thaw the processed specimens at room temperature and then continue with step in section B. To complete specimen preparation and amplification on day 1 and detection on day 2, perform sections a, b, c on day 1 and store the denatured amplicon at 2 to 8°C for up to one week. Continue with section D on day 2.

Reagent Preparation

(1.) Preparation working Master Mix by adding 100 microliters of Manganese Solution to one tube of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10-15 times (this is "working master mix"). The pink dye in the Manganese Solution is for visual confirmation that the Manganese Solution has been added to the Master Mix. Leftover Manganese Solution should be discarded.

(2.) Place the appropriate number of PCR reaction tubes, 12 tubes to a row, into a MicroAmp sample tray and lock the tubes in position with the tube retainer.

(3.) Pipette 50 microliters of working master mix into each PCR tube using a micropipettor with an aerosol resistant tip. Discard leftover working master mix.

(4.) Place the microtube tray in a plastic zip-lock bag and store the tray at 2 to 8°C until the specimen preparation is completed. **Amplification must begin within 4 hours of the preparation of the working master mix**.

(5.) If performing the specimen preparation in the same area, remember to clean the area. Remove gloves and dispose of them properly. Remove lab coat and/or any other protective garments.

Specimen and Control Preparation

(1.) Prepare 70% ethanol, volumes depend on if using 95% ethanol or 90% ethanol.(for 12 tests, mix 11ml of 95% ethanol and 4ml of deionized water.

(2.) Prepare working Lysis Reagent as follows:

Warm the cold Lysis Reagent until the crystals have dissolved. Mix for at least ten seconds prior to use to ensure that it is thoroughly mixed.
Add 100ul QS. The lysis reagent will now be pink.

(3.) Label a 2.0ml screw cap microcentrifuge tube for each sample, including one tube for each kit control and/or standard.

(4.) Thaw plasma specimens and if used, standards to room temperature and vortex each tube for 3-5 seconds.

(5.) Briefly centrifuge each specimen tube in order to collect the sample into the base of the tube. Do not contaminate gloves while manipulating specimens.

(6.) Dispense 600µl of working Lysis Reagent into each labeled microcentrifuge tube. Check that the working lysis reagent is pink to confirm that the QS was added to the lysis reagent.

(7.) Add 200µl of plasma or standard to each appropriately labeled microcentrifuge tube containing the working Lysis Reagent. To the controls, add 200µl of NHP and 50µl of the controls to the microcentrifuge tube. Re-cap the tube and vigorously vortex for 3-5 seconds.

(8.) Incubate the tubes for 10 minutes at room temperature.

(9.) Remove the cap from each tube and add 800µl of 100% Isopropanol to each tube. Re-cap the tubes and vigorously vortex them for 3-5 seconds.

(10.) Place an orientation mark on each tube and place the tubes into the microcentrifuge with the orientation marks facing outward, so that the pellet will align with the orientation marks. Centrifuge the tubes at maximum speed (at

least 12,500 x g) for 15 minutes at room temperature. Upon completion of the centrifugation carefully remove each tube from the rotor,

(11.) Beginning with the control tubes, carefully draw off the supernatant, without disturbing the pellet (which may not be visible), using a fine tip, disposable transfer pipette. **Remove as much liquid as possible without disturbing the pellet: slide the pipette down the inside of the tube along the side opposite the pellet while drawing off the liquid. Maintain a continuous negative pressure with the transfer pipette as you draw off the liquid**.

(12.) Add 1ml of 70% ethanol to each tube, re-cap, and vigorously vortex 3-5 seconds.

(13.) Place the tubes into the microcentrifuge with the orientation marks facing outward and centrifuge the sample at maximum speed for 5 minutes at room temperature.

(14.) Carefully remove the tubes from the centrifuge rotor and aspirate the supernatant as described as above. The pellet should be clearly visible at this time. **Remove as much of the supernatant as possible! (Do this twice). Residual ethanol can inhibit the amplification**.

(15.) Add 400µl of Specimen Diluent, re-cap, and vigorously vortex for at least 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

(16.) Amplify the processed specimens within 2 hours of preparation or store frozen at -20°C for up to one week.

(17.) Preparation for amplification: If previously frozen, warm extracted specimens to room temperature, vigorously vortex each tubes for at least 10 seconds.

(18.) Pipette 50µl of extracted specimens, controls to the appropriately designated reaction tubes which were previously prepared using a micropipettor with a plugged tip. Use a new tip for each specimen and control.

(19.) Securely cap each tube and using the MicroAmp capping tool seal the tubes.

(20.) Transfer the tray with sealed tubes containing the processed specimens and controls in working master mix to the Amplification/Detection area.

(21.) Decontaminate work area with a 1:10 dilution of bleach. Follow by cleaning area with propanol.

Reverse Transcription and Amplification

NOTE: Turn on the GeneAmp PCR System 9600 thermal cycler at least 30 minutes prior to beginning amplification.

(1.) Place the reaction tray into the thermal cycler sample block. **Make sure that the notch in the reaction tray is at the left of the block, and that the rim of the tray is seated in the channel around the block**.

(2.) Make certain that the cover knob is turned completely counterclockwise and slide the cover forward.

(3.) **Turn the cover knob clockwise until hand tight**.

(4.) Program the GeneAmp System 9600 thermal cycler as follows:

(5.) Hold 2 minutes at 50°C

(6.) Hold 30 minutes at 60°C

(7.) 8 cycles 10 seconds at 95°C, 10 seconds at 52°C, 10 seconds at 72°C

(8.) 23 cycles 10 seconds at 90°C, 10 seconds at 55°C, 10 seconds at 72°C

(9.) Hold 15 minutes at 72°C

(10.) In the CYCLE programs the ramp time and allowed setpoint error should be left at the default settings of 0:00(which is the maximum rate) and 2'C.respectively. Link the 5 programs together into a METHOD program.

(11.) Start the method program. The program runs for approximately one hour and 30 minutes.

(12.) Remove the reaction tray form the thermal cycler beyond the end of the final Hold period. Do not allow the tubes to remain in the thermal cycler beyond the end of the final Hold period and do not extend the final HOLD program beyond 15 minutes. **Do not bring amplified DNA into the other areas. The amplified material should be considered to be significant potential source of DNA contamination.**

(13.) Remove the caps from the reaction tubes carefully so as to avoid aerosols of the amplification products. Immediately pipette 100µl of MONITOR Denaturation Solution into each reaction tube using a multichannel Pipettor, and mix carefully pipetting up and down at least 5 times. Preferably use the AMPLICOR Electronic IMPACT Pipettor set on Program 1 (see separate procedure).

(14.) The detection amplicon can be held at room temperature no more than 2 hours before proceeding to the detection reaction. If the detection reaction can not be performed within this time, re-cap the tubes and store the denatured amplicons at 2 to 8°C for up to one week.

Detection

(1.) Warm all reagents and amplicons to room temperature prior to proceeding with the detection.

(2.) Prepare a sufficient amount of working Wash Solution (dilute 1 part of the 10X with 9 parts of distilled or deionized water). This working Wash Solution is stable for up to 2 weeks at room temperature.

(3.) Allow the microwell detection plate to warm to room temperature before removing it form its foil pouch.

(4.) Add 100µl of MONITOR Hybridization Buffer to each well using Program 2 on the IMPACT Pipettor.

(5.) Add 25µl of the denatured amplicons to the wells of row A of the detection plate, mix up and down 10 times (twice). Use aerosol resistant plugged pipette tips. Make serial 5-fold dilutions in wells B through F as follows: transfer 25µl from row A to B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25µl. Discard pipette tips. This may be done using the IMPACT Pipettor Program 3 two times, where 25µl is transferred and mixes by pipetting 60µl up and down 10 times, and aspirates 25µl.

(6.) Add 25µl of the denatured amplicons to the wells of row G of the detection plate in the same manner as described in step 5. Mix as described in step 5 and transfer 25µl from row G to row H, again, mix as described in step5; remove and discard 25µl from row H along with the pipette tips.

(7.) Cover the plate and incubate it for 1 hour at 37°C (± 2°C).

(8.) Wash the entire detection plate 5 times with the working Wash Solution and an automated microplate washer. The microplate washer should entirely fill each well (400-450µl), allow each row (or column) soak for 30 seconds, then aspirate the entire contents of each well before proceeding to the next cycle.

(9.) Add 100µl of Avidin-HRP conjugate to each well, cover plate and incubate for 15 minutes at 37°C (±2°C).

(10.) Wash the plate as described in step 8.

(11.) Prepare the working Substrate solution by mixing 4 parts of substrate A with 1 part of substrate B. Protect the working solution from direct light.

(12.) Pipette 100µl of the working Substrate solution to each well and allow the color (light blue to dark blue) to develop for 10 minutes at room temperature in the dark.

(13.) Add 100µl of Stop Reagent to each well (blue color will turn to a yellow color).

(14.) Measure the optical density at 450 angstroms within 10 minutes of adding the Stop Reagent otherwise less dilute wells will precipitate resulting in a lower OD.

(15.) Decontaminate work area with a 1:10 bleach solution.

CALCULATIONS:

MANUAL CALCULATIONS

(1.) For each specimen, control or standard, choose the appropriate HIV well, as follows:

a. The HIV wells in rows A through F represent neat and 5-, 25-, 125-, 625-, and 3125-fold serial dilutions of the amplicons, respectively. The absorbance values should decrease with the serial dilutions, with the highest value for each test in row A and the lowest value in row F.

b. Choose the well where the raw OD is in the range of 0.200 to 2.0 OD units. If more than 1 well is in this range, choose the well with the larger dilution factor (i.e., the smaller OD).

c. If any of the following conditions exist see Unexpected Results, below: all HIV OD values <0.200; all HIV OD values >2.0; HIV OD values are not in sequence (i.e., the OD values do not increase from well A to well F).

(2.) Subtract a background value of 0.070 OD units from each of the selected HIV OD values.

(3.) Calculate the "total HIV OD" by multiplying the value derived in step 2 by the dilution factor associated with that well.

(4.) For each specimen, control or standard, choose the appropriate QS well, as follows:

a. The QS wells in rows G and H represent neat and 5-fold dilutions of the amplification products, respectively. The absorbance value in row G should be greater than the value in row H.

b. Choose the well where the OD is in the range of 0.300 to 2.0 OD units. If both wells are in this range, choose well H.

c. If one of the following conditions exist, see Unexpected Results, below: both QS OD values <0.300; both QS OD values >2.0: QS OD values are not in sequence (i.e., the OD values do not decrease from well G to well H).

(5.) Subtract a background value of 0.070 OD units from each of the selected QS OD values.

(6.) Calculate the "total QS OD" by multiplying the value derived in step 5 by the dilution factor associated with that well.

(7.) Calculate HIV-1 RNA Copies/mL plasma as follows:

HIV-1 RNA Copies/mL Plasma = ("total HIV OD"/"total QS OD") X Input QS Copies per reaction X 40

Computerized (Manual – LDMS) Calculations

(1.) On LDMS System, go to the assay module.

(2.) Click on the (+) sign next to Viral Load RNA from the Assay lists box.

(3.) Then click on the New Run/Not Setup button on upper right side of Search Criteria box.

(4.) Click on the Select button at the bottom of the Assay Selection screen. The Filters/Criteria screen appears.

(5.) At this time it is unclear whether the Filers/Criteria screen will be used. This section is used to pull pending viral loads from the database by using specific parameters. . At this point we will not be using the button "All Pending Specimens" because the list pulled would be too numerous to look through. The best suggestion at present to find specimens is to select the group from the Group combo box. Then narrow your search, create query statements using the Field, Operator and Value combo boxes.

(6.) Click the Find Specimens button at the bottom of the screen. This will take you to the Specimens Found screen. Use the shift or ctrl keys to select each specimen you want to place in the assay or use the buttons at the bottom of the Specimens Found screen. After selecting specimens, click the Add to Plate button at the bottom of the screen. LDMS will automatically move to the Plate Preview screen and load the chosen specimens onto the plate in the order determined by the user.

(7.) Repeat the process from step five if more than one group needs to be put on the run.

(8.) In the Plate Preview screen the user can add and delete plates to run; move, delete add and modify specimens; move, add, and modify controls; save the template to run later; click the Run Now button to run the assay.

(9.) After saving the assay od's from the LDMS Remote Reader Software, it can be read into the LDMS database and merged with the template previously set up. Put the disk into the A: drive. Go to the Assay module again.

(10.) Click the Viral Load RNA.

(11.) Click on the Runs Not Performed button.

(12.) Enter the run id of your assay in the Run Id field.

(13.) Click on your assay to select it, then click on the Select button at the bottom right of screen. The Preview tab will activate.

(14.) Click on the Preview tab, then click the Run Now button the bottom of the screen.

(15.) The input Copies dialog box will appear. Enter a value in the edit field, then click on the ok button on the dialog box.

(16.) A file dialog box will appear. Open the A:/devdata folder, then click on your file.

(17.) Click on the Open button on the File dialog box, and LDMS will begin reading the raw data from the disk in your A: drive.

(18.) When the LDMS is finished reading the assay data from your remote reader disk, the Results screen will appear in Plate Results view displaying the raw data from your remote assay run. Click on the Calculated Results button to view results on a specimen by specimen basis.

RESULT CRITERIA

(1.) If all of the HIV wells have OD values less than 0.200, but the QS wells have the expected values, use 0.200 as the HIV OD, calculate the result, and report the result as "Not detected, less than" the calculated value.

(2.) If all the HIV wells have OD values greater than 2.0, but the QS wells have the expected values, either an error occurred in the test, or the HIV copy number is above the dynamic range of the assay. Report the result as "Not determined". Repeat the entire assay, making a 1:50 dilution with HIV negative human plasma. Calculate the results as above and multiply by 50.

(3.) If the HIV wells do not follow the pattern of decreasing OD values from well A to well F, and error in dilution may have occurred. Examine the data according to the following criteria to determine if an error occurred. If an error occurred, report the result the as "Not determined" and repeat the entire assay including specimen preparation; otherwise, calculate and report the result as described above:

a. The OD values for HIV wells should follow a pattern of decreasing OD values with increasing Dilution Factor 9 (i.e., from well A to F), expect for well that are saturated and wells with background OD values.

b. In reactions containing high HIV-1 RNA copies per ml, wells A, B, and C can become saturated turning a greenish – brown color prior to the addition of Stop Solution and a brown color after addition of Stop Solution, resulting in lower OD. These results are valid even though the HIV wells do not have decreasing OD values from wells A through F.

c. In reactions containing low HIV-1 RNA copies per ml, wells B through F may contain background OD values. Such tests are valid even thought he HIV wells do not have decreasing OD values from well A through F. Wells with OD values (>2.3) may be saturated and wells with very low OD values (<0.1) are close to background. These wells may not follow a pattern of decreasing OD values from well A to well F.

d. All well with OD values </=2.3 and >/=0.1 should follow a pattern of decreasing OD values from well A to well F. If OD values do not follow a pattern of decreasing OD values from A to F then an error occurred.

(4.) If both QS wells have OD values less than 0.300, either the processed sample was inhibitory to the amplification, or the RNA was no recovered from the sample. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.

(5.) If both QS wells have OD values greater 2.0, an error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.
(6.) If the absorbance well H is greater than the absorbance of well G, and error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.
(7.) The assay is used for research purposes and for use in clinically monitoring viral loads.
(8.) Examples of unexpected results below:

ROW	Dilution Factor	Example 1	Example 2	Example 3
A	1	2.610	2.564	0.812
B	5	2.461	2.684	0.0161
C	25	3.112	2.432	0.055
D	125	2.668	1.032	0.064
E	625	2.984	0.287	0.079
F	3125	1.568	0.074	0.052
Interpretation:	Very high titer specimen. Not an error.	High titer specimen. Not an error.	High titer specimen. Not an error.	Low titer specimen. Not an error.

REPORTING RESULTS:

Results will be reported on the data fax form.

PROCEDURE NOTES:

(1.) Heparin should not be used as an anticoagulant as it inhibits PCR.
(2.) Residual ethanol left on the pellet will inhibit the amplification.
(3.) Due to the high analytical sensitivity of this test and the potential for contamination, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures.
(4.) All reagents should be closely monitored for purity. Discard any reagents that may be suspect.
(5.) Workflow in the laboratory should proceed in a uni-directional manner, beginning in the reagent preparation area, then the specimen preparation area, onto the Amplification/detection area.
(6.) Supplies should be detected to each activity and must not be used for other activities or moved between areas. Equipment and supplies used for reagent preparation/specimen preparation activities must not be used for pipetting or processing amplified DNA or other sources of target DNA.
(7.) Gloves must be worn in each and changed before leaving that area.
(8.) Good laboratory technique is essential to the proper performance of the assay.

LIMITATIONS OF PROCEDURE:

(1.) The presence of AmpErase in the AMPLICOR HIV-1 MONITOR Master Mix reduces the risk of amplicon contamination. However, contamination from HIV positive controls and HIV positive clinical specimens can be avoided only by

good laboratory practices and careful adherence to the procedures specified above.

(2.) Use of this product should be limited to personnel trained in the techniques of PCR.

(3.) Only the Perkin-Elmer GeneAmp PCR System 9600 or GeneAmp 2400 thermal cyclers can be used with this product.

(4.) Monitoring the effects of antiretroviral therapy by serial measurements of plasma HIV-1 RNA has only been validated for patients with baselines viral loads >= 25,000 copies/ml.

The performance of the Amplicor HIV-1 Monitor Test has only been validated with HIV subtype B specimens.

_____ _____

Signature of Person Administering Consent Date

Appendix D: PBMC Isolation and Cryopreservation /ELISPOT Assay Protocol

Please note: This appendix is NOT the final HCL protocol manual for this study. It is designed to familiarize readers with technologies that have not previously been included in HIVNET or HPTN studies. Final protocol manuals will be generated and distributed prior to the initiation of the studies.

General Lab Supplies and Reagents Suggested for Lymphocyte Isolation and Cryopreservation:

Supply	**Description**	**Suggested Vendor/Catalog #**
50 ml tubes:	Polypropylene disposable tube (**DO NOT USE POLYSTYRENE)**	Fisher Scientific/05-539-6
15 ml tubes	Polypropylene disposable tube (**DO NOT USE POLYSTYRENE**)	Fisher Scientific 05-5395-5
Pipettors	20, 200, 1000 µL pipettors recommended	Ranin/P-20, P-200, P-100
Pipette Tips	250 µL capacity in racks of 100/rack	Ranin/ RT-20 *
Pipette Aid	NOTE: Available in 110V, 220V	Drummond/VWR/4-000-111(110V), 4-000-220 (220V)
Pipettes	2 ml plastic individually wrapped-sterile 10 ml plastic individually wrapped-sterile 25 ml plastic individually wrapped-sterile	Fisher/29442-418 Fisher/29442-430 Fisher/29442-436
Hemocytometer	Hausser Scientific for Phase Microscope	VWR/15170-079
Hemocytometer Cover Slips	Octagonal cover slip	VWR/151-70-296
Trypan Blue	For assessment of cell viability	Life Technologies/152250061
Ficoll-Paque™	Store in dark bottle at room temperature	Amersham-Pharmacia/17-1440-03
RPMI 1640 1X with Glutamine	Liquid Media 10 x 500 mls	Life Technologies/11875119
Penicillin /Streptomycin	Store in frozen aliquots of 5-10mls at –20^{o}C	Life Technologies catalog/15140-122. Stock solution is 100X.
Fetal Bovine Serum	Store at –70^{o}C	Price compare

DMSO	Six month shelf life	J.T. Baker/9224-01
10X PBS	Dilute to 1X with ddH_20	Life Technologies catalog # 14190-144
Nalgene "Mr. Frosty"	Used for control rate freezing. Store at 4^oC when not in use.	Curtis Matheson Scientific/288-383
Cryogenic Freezing Vials	2 mL polyethylene vials with screw cap	Fisher Scientific/12-565-171N
Purple Top EDTA Tubes	10ml purple top EDTA vacutainer tubes	VWR/VT6457
Green Top Tubes	10 ml Sodium Heparin vacutainer tubes	VWR/VT6480
96-well plate	Round Bottom styrene plates	VWR 29442-398

REAGENTS TO MAKE

Solution Name:	**Recipe:**	**How to Make:**
FCS		Heat FCS at 56°C for 30 minutes to inactivate complement proteins
Culture Media (R-10)	RPMI/ 10% FBS/1X Pen/Strep.	To 500 ml bottle of RPMI 1640 1X Media, add 56 mls of FCS and 5.6 mls of 1X Pen/Strep. Store at 4^oC in dark.
Culture Media (R-1)	RPMI/ 1% FBS/1X Pen/Strep.	To 500 ml bottle of RPMI 1640 1X Media, add 5 mls of FCS and 5mls of 1X Pen/Strep. Store at 4^oC in dark.
Freezing Media (FM):	90% heat-inactivated FCS/10% DMSO.	Keep at 4^oC in dark bottle for up to one month.
Washing Media (WM)	PBS/1%FCS/1%Pen-Strep	To 500ml bottle of PBS add 5 ml of FCS and 5 ml of Pen-Strep.

LYMPHOCYTE ISOLATION AND CRYOPRESERVATION METHOD:

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NOTES:

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- **Do not refrigerate or freeze vacutainer tubes. If they arrive cold (from weather-related transport), allow them to come to room temperature.**
- **Store tubes at room temperature prior to processing.**

- **Make sure tubes are adequately mixed by inverting the tubes gently several times prior to ficoll procedure.**
- **Both Ficoll and DMSO are toxic to cells. It is necessary to work quickly and wash well to minimize cell loss.**

Supplies needed:
50ml sterile conical polypropylene tubes.
Sterile 25 and 10ml pipettes
Ficoll Hypaque (1.077-1.080 g/ml)
Wash media
Freezing media
Sterile Cryovials
96-well counting plate/small tubes for counting cells
Mr. Frosty
Hemocytometer
P20 pipettor, tips

1) 50 ml tubes containing ficoll can be prepared in advanced and store at room temperature. The blood:ficoll ratio should prepared according to the manufacturer's instructions. Blood can be diluted 1-1.5 with WM to bring the volume up when necessary.

2) Remove blood from vacutainer tube using a 10ml pipet, and carefully over-layer the blood onto the Ficoll. Do not disturb or mix tube once Ficoll has been added. This will destroy the gradient layer.

3) Centrifuge for 25 minutes at 400 x g at room temperature.

4) Carefully pipette buffy coat layer with a 10 ml pipette being careful not to take any of the RBC pellet (should pull up about 7-8 ml). Pipette cells into a fresh 50 ml tube, and bring up to 45ml with WM.

5) Centrifuge for 15 min at 400 x g.

6) Discard supernatant. Resuspend cell pellet gently by adding 5 ml of WM and mixing well with same pipette. Add additional 35 ml of WM, cap tube and invert several times.

7) From the well-mixed cell suspension take out 20µl using P-20 pipettor and put in 96-well counting plate. Re-cap tube and centrifuge for 10 min at 400 x g.

8) While cells are spinning, count cells:

Cell counting:

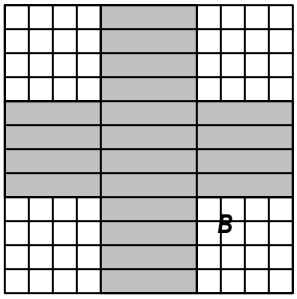
The following steps are meant to be used as a guideline for lymphocyte counting using a hemocytometer. Counting lymphocytes using a hemocytometer is

recommended over the Coulter Counter method so that cell viability and morphology can be viewed by eye.

- After resuspending cells, take a small amount (20µl) of the cell suspension and transfer to a well in the 96 well plate or a small tube.
- Add an equal volume of Trypan Blue dye to dilute the cells 1:2. Mix the cell suspension well and add to the hemocytometer by placing the tip of pipette to the space between the groove in the hemocytometer and the cover slip. Push the pipette plunger just enough to let the cell suspension be taken up by capillary action until the counting grid is just covered. DO NOT add too much liquid.
- When using Trypan Blue exclusion dye, live cells are white and dead cells are blue. **Note:** it is sometimes difficult to distinguish red blood cells (RBCs) from lymphocytes.
- Using the picture below, count cells that are found in squares A and B. You should count cells within the triple line boundary; cells can be touching this boundary, but should not cross the boundary.
- After the both A and B squares have been counted, add the two together and multiply that by 10^4. This calculation will give you the number of cells per ml. To obtain total cell number, multiply the number of cells/ml x total volume of cell suspension.

- **Example:** if you count 34 cells in A and 25 cells in B, them $34+25=59x10^4=5.9x10^5$ cells/ml of sample. If the sample is in 10 mls of media, then the total number of cells is $5.9x10^6$.

- Clean the hemocytometer and cover slip with bleach and 70% ethanol.



9) **Resuspend pellet to a final cell concentration of 10^7 cells/vial in FM.**

10) WORK QUICKLY AT THIS POINT TO GET CELLS IN FREEZER!!

11) Aliquot 0.5 to 1.0 mL of the cell suspension per cryovial. Be sure cryovial caps are securely tightened so that liquid nitrogen does not leak in. This can cause the vial to burst upon thawing.

12) **Immediately** place cryovials in isopropanol freezing container (i.e. "Mr. Frosty") and transfer to a -70°C freezer. Alternatively, place cryovials in a Cryomed controlled rate freezing chamber, lowering the temperature at -1°C per minute to -70°C.

13) Transfer cryovials to liquid nitrogen after 24 hours at -70°C.

Quality Control for Lymphocyte Isolation

- During the lymphocyte isolation procedure, the cells must go through a series of washes to remove residual Ficoll, which is toxic to cells.

- The QC for this step will be recorded on the specimen collection form as the lymphocyte viability (# live cells / # live cells + # dead cells).

- This will determine the quality of blood layering onto Ficoll, quality of isolation and collection of a buffy coat layer, and quality of washing Ficoll from cells. All of these conditions are necessary to prepare cells for optimal cryopreservation.

THAWING PROCEDURE:

1. Transfer cryovial from liquid nitrogen to a 37°C water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.

   WARNING: Some cryovials have been reported to explode during the thawing process. To minimize this risk, use only unbreakable polyethylene vials for storage in liquid nitrogen (see above under materials/reagents). If polypropylene tubes are used, they should be sealed in cryoflex tubing.

2. Hold the cryovial in the surface of the water bath with an occasional gentle "flick" during thawing. Do not leave cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly). When a small bit of ice remains in the cryovial, transfer the cryovial to the biosafety hood. Dry off the outside of the cryovials before opening to prevent contamination.

3. Add R1 drop wise into the cryovial containing the cell suspension up to a volume which doubles the original volume (e.g., add 1 mL of media to a vial containing 1 mL cell suspension).

4. Transfer the cell suspension to a 15 ml conical bottom centrifuge tube containing 8 mL of warmed R1; wash twice by centrifugation, gently resuspending the cells between washes.

5. Determine cell number, record both alive and dead, to determine cell viability. Resuspend cells at desired concentration for assay to be used.

ELISPOT ASSAY for detection of secreted IFN-g

Products needed for the assay:

Products	**Company**	**Catalog #**
96 well/plate MILLIPORE Multiscreen Plates	MILLIPORE	MAHAS4510
Primary AB: Anti-IFN? Mab 1D1K	MABTECH	3420-3
Secondary AB: Anti-IFN? Mab 7B61 Biotinylated	MABTECH	3420-6
Vectastain	Vector Laboratories	PK6100
Stable DAB	Research Genetics	750118
PBS w/o Ca and Mg	GIBCO	14190-136
Tween- 20	Biorad	170-6531
RPMI 1640	Life Technologies	11875119
DMSO	GIBCO	61870-036
PHA-P 5mg/vial	SIGMA	D-2650
10X PBS	Gibco BRL	14200-075
Plate sealer (to hold plate membranes)	Millipore	MATA HCL 00
Filters (to punch out membranes)		
Media		
RPMI 1640 supplemented with 10% FCS (R10)		
RPMI 1640 supplemented with 20% FCS (R20)		
1X PBS-Tween supplemented with 0.1% Tween 20 (PBS-T)		
DMSO		

Procedure

Day 1:

1. Dilute primary ab for plating: The stock solution of the primary anti-IFN? monoclonal antibody is 1mg/ml. The working concentration is 10ug/ml. Add 10ul of the stock to every 1 ml of 1X PBS (eg 50 ul anti-IFN? and 5 mls of PBS to coat one plate). Pipet 50ul into each well of HA plates.
2. Store at 4°C overnight wrapped to prevent evaporation.

3. Thaw PBMC to be tested and record viability and recovery. Resuspend at 2-4x10^6 cells per ml of R-20 in a 50ml conical tube with the cap loosened. Do not exceed 5mls/50 ml tube. Let the cells rest overnight in CO_2 incubator.

Day 2

1. Wash antibody coated plates 4x with 1X PBS.
2. Block plates with R10 for at least 30 minutes at 37°C or until the cells are ready to be plated.
3. Prepare the cell dilutions: Count cells (record live and dead cells), spin cell suspension at
   400 x g and resuspend the cells at 1x10^6 cells/ml. This will give 1x10^5 cells/100ul.
4. Prepare the peptide pools/matrix dilutions: TBA
5. ***Discard the blocking medium from the plates.***
6. Pipet 100ul of the 1x10^6 cells/ml to each well. Final concentration of cells is 10^5 per well.
7. Add 100ul of R-10/DMSO to each of the mock wells.
8. Add 100ul of the R-10/PHA/DMSO dilution to each of the PHA wells.
9. Add 100 ul of the peptide dilutions to the peptide wells.
10. Incubate for a minimum of 12h or up to 18h at 37°C in 5%CO_2.

Day 3

1. Discard the cell suspension
2. Wash by pipetting 100ul of PBS-T (PBS containing 0.1% Tween 20 – Sigma) into each well. Discard the PBS-T by flicking plate into large container in hood. Blot plate on paper towel. Repeat 4x.
3. Add 50µl/well of diluted secondary antibody (dilute stock antibody 1:1000 in 1X PBS to 1µg/ml) Incubate at room temperature for 2h.
4. Thirty minutes before the incubation has ended, make the Avidin bound Biotinylated HRP (Vectastain Elite Kit) by adding 2 drops of reagent A + 2drops of reagent B to 5 ml PBST and allow to stand at RT for 30 min to let complex form).
5. Wash the plate 4x in PBS-T.

6. Add 50µl to each well of Avidin bound Biotinylated HRP and incubate for 1h at room temperature.
7. Pipet out an appropriate amount of stable DAB into a conical tube and let stand at room temperature (100µl/ well; 10mls/plate).
8. Wash the plates 4x in PBS-T. Add 100µl / well of stable DAB /well and incubate for no more than 2 minutes.
9. At 2 minutes, discard the Stable DAB from the wells and quickly 1dd 100µl/well of tap to stop the reaction. Discard. Rinse plate under running tap water several times to prevent over-development.
10. Count the spots with a stereomicroscope (Stemi 2000 stereo microscope, Carl Zeiss, Inc. New York) under magnifications of 20-40x.
11. Only spots with a fuzzy border and a brown color are to be counted. (A positive test is 5 spot forming colonies per 10^5 cells after deducting the control).
12. After counting, peel off the plastic backing on the plate and stick a sheet of Elisa plate sealer onto the membranes. Using a filter, punch out each well individually to insure that the membrane does not break when it is transferred to the sticky plate sealer. Either place the plate sealer cover back onto the plate sealer or store in a page protector until transport to the core laboratory for computer counting.

Appendix IV

HPTN 034B: HIV Prevention Preparedness Study Protocol Pune, India

HPTN 034B HIV-1 Exposed, Uninfected Laboratory Sub-study

HPTN 034 HIV Incidence and Participant Retention Protocol

HPTN EXPOSED-UNINFECTED
HPTN 034B LABORATORY SUB-STUDY

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Dr. Srikanth Tripathy
Dr. Madhur Thakur
Dr. Smita Kulkarni

TABLE OF CONTENTS

APPENDICES:

ABBREVIATIONS

Ab	antibody
AACTG	Adult AIDS Clinical Trial Group
AIDS	acquired immune deficiency syndrome
bDNA	branched deoxyribonucleic acid
CL	Core Laboratory
CD	cluster designation
CDC	Centers for Disease Control and Prevention
CM	culture medium
env	envelope
FHI	Family Health International
FBS	fetal bovine serum
FACS	fluorescent activated cell sorter
FM	freezing medium
gag	group associated antigen
gp	glycoprotein
HIV-1	human immunodeficiency virus type 1
HIVNET	HIV-1 Network for Prevention Trials
HLA	Human Leukocyte antigen
HPTN	HIV Prevention Trials Network
HTPU	HIV Prevention Trials Unit
IFNγ	interferon-gamma
MHC	major histocompatibility complex
min	minutes
ml	milliliter
MRC	Medical Research Council
Nef	negative factor
NIAID	National Institute of Allergy and Infectious Diseases
RT	reverse transcriptase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
STD	sexually transmitted disease
WM	wash medium

HPTN 034B
HPTN EXPOSED-UNINFECTED LABORATORY SUB-STUDY

A Study of the HIV Prevention Trials Network

Sponsored by:

Division of AIDS (DAIDS)
US National Institute of Allergy and Infectious Diseases (NIAID)
US National Institutes of Health (NIH)

We, the Principal Investigators, agree to conduct this study in full accordance with the provisions of this protocol. I agree to maintain all study documentation for a minimum of five years from the end of the study, unless directed otherwise by the HPTN Coordinating and Operations Center (CORE). Publication of the results of this study will be governed by HPTN and DAIDS policies. Any presentation, abstract, or manuscript will be made available by the investigators to the HPTN Manuscript Review Committee and DAIDS for review prior to submission.

We have read and understand the information in this protocol and will ensure that all associates, colleagues, and employees assisting in the conduct of the study are informed about the obligations incurred by their contribution to the study.

Name of Principal Investigator _____

_____ _____
Signature of Principal Investigator Date

Name of Principal Investigator _____

_____ _____
Signature of Principal Investigator Date

HPTN 034 B
HPTN EXPOSED-UNINFECTED LABORATORY SUB-STUDY
PROTOCOL SUMMARY

Design:	Laboratory Sub-study for HPTN 034
Study Sites/Population:	Adult men and women who have been identified as HIV-1 uninfected sexual partners of recently HIV-1 infected participants in the HPTN 034A sub-study
Study Duration:	The sub-study is designed to run for the duration of the entire HPTN 034 funding period. Participants will be continuously enrolled and followed for up to two years, as funding allows.
Primary Objectives:	**(a)** To determine if the detection of an HIV-1 -specific immune response in exposed uninfected individuals correlates with risk of subsequent HIV-1 infection. **(b)** To determine if immunologic genetic characteristics of exposed uninfected individuals correlates with risk of subsequent HIV-1 infection.
Primary Endpoints	**(a)** HIV-1 antibody seroconversion **(b)** ELISPOT measurement of HIV-1 peptide-specific T lymphocytes **(c)** HLA-Class I MHC alleles
Secondary Objectives:	**(a)**To determine if the detection of an HIV-1 -specific immune response in exposed-uninfected individuals correlates with the HIV-1 viral set point in individuals that subsequently become HIV-1 infected. **(b)** To compare the epitope specificity of the HIV-1-specific immune response of exposed-uninfected individuals with that of their recently infected partners. **(c)** To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples.
Secondary Endpoints:	**(a)** Median HIV-1 viral load between 6 and 12 months after estimated time of infection, for participants that HIV-1 seroconvert. **(b)** HLA restriction and minimal peptide sequence of responding T lymphocytes from exposed-uninfected participants. **(c)** Enrollment and retention of study participants and their acutely infected partners enrolled in the 034A HPTN Early Infection Study.

1.0 INTRODUCTION

1.1 Background

AIDS, caused by infection with the human immunodeficiency virus 1 (HIV-1), is associated with enormous morbidity and mortality worldwide. Current estimates are that there are nearly 35 million people infected with HIV-1 globally, and that there are 15,000 new infections occurring daily (1). Developing countries shoulder nearly ninety five percent of all new HIV-1 infections. Although recent advances have been made in antiretroviral therapy for HIV-1 infection, there is no cure for AIDS, and the expense of drug therapy is prohibitive for most of the population. For this reason, clinical HIV-1 prevention trials sponsored by NIAID and the HPTN and HVTN are ongoing and in development. These trials include prevention through antiretroviral drugs, microbicides, behavioral approaches, barrier and other contraceptive/STD prevention methods, vaccines (in perinatal settings), chemoprophylaxis, treatment of sexually transmitted diseases, and combined approaches (2).

Despite important advances in understanding and identifying critical host and viral factors that can influence acquisition of HIV-1 infection, viral load and disease progression, the goal of translating this information into effective prophylactic treatments and vaccines has not been achieved. Toward this end, many immunologic studies have been done on cohorts of individuals that exhibit relative resistance to infection with HIV-1 despite multiple high-risk exposures. Standard HIV-1 IgG ELISA assays and Western blots are negative in these subjects, as are sensitive HIV-1 RNA PCR assays in the peripheral blood. Because there remains the possibility that persistent infection has been established in these cases, but viremia has been controlled to levels below the limits of detection these individuals are often referred to as Highly Exposed, Persistently Seronegative (HEPS). Resistance to HIV-1 infection has been observed in sex partners of HIV-1 infected persons (3-7), infants born to HIV-1 infected mothers (8-11), health care workers occupationally exposure to HIV-1 -contaminated body fluids (7;12) and commercial sex-workers (CSWs) (3;4). It has been hypothesized that protection against human immunodeficiency virus HIV-1 infection may result from either acquired host immunity, inheritance of a dysfunctional HIV-1 co-receptor (such as CCR5), a low or attenuated virus inoculum, or host genetic factors such as HLA genotype (13;14). Thus far, no evidence exists for a significant role for CCR5 Δ32/Δ32 mutation in non-Caucasian North American populations (6;7;15). Because more than 80% of HIV-1 infections world-wide occur through heterosexual transmission, it has been hypothesized that the mechanism of protection in these individuals is mediated, in part, by cellular immune mechanisms present at mucosal sites of exposure (16).

The association of HIV-1 -specific CTL with non-progressive disease in HIV-1 infected persons, as well as the increased frequency of certain HLA class I alleles in HEPS populations, has led several groups to look for HLA class I restricted, HIV-1 -specific CTL in these groups(17-24). HIV-1 -specific CTL have now been described in the peripheral blood of HEPS (16). Studies by several investigators have shown that HIV-1 -specific $CD8^+$ T cells can be detected in the peripheral blood of 30-60% of HEPS tested. These $CD8^+$ responses have been measured both in conventional CTL lysis assays (20) as well as IFN-γ ELISPOT assays (21). Responses to multiple HIV-1 antigens were detected including env, gag, pol and nef. Interestingly, HIV-1 -specific CTL have recently been demonstrated in the genital tract, the

likely site of viral exposure, in a subset of a HEPS cohort in Nairobi (21). It has been hypothesized that a primed CTL response at the site of exposure would be more relevant in protection against incident HIV-1 infection than in blood, where HEPS CTL responses have been previously described (21). These $CD8^+$ cells were most likely intraepithelial memory T lymphocytes generated in secondary lymphoid organs that localize to epithelial sites upon re-exposure to antigen (25). Overall, these responses (both mucosal and peripheral) tended to be lower and not as durable as those seen in seropositive individuals. Nonetheless, the detection of HIV-1 -specific CTL in the genital tract of these HEPS suggests that these responses may be important in mediating protective immunity against heterosexual HIV-1 acquisition.

Resistance to infection with HIV-1 based on a noncytolytic $CD8^+$ T cell-mediated mechanism has also been reported. Stranford et al described a $CD8^+$-mediated, noncytolytic anti-HIV-1 activity that was seen in the vast majority of individuals most recently exposed to the virus (within 1 year), and was potent enough to inhibit *in vitro* infection of subject's PBMC (26). Paxton et al, showed that resistance to infection of $CD4^+$ lymphocytes from HEPS was associated with the activity of C-C chemokines such as RANTES, MIP1α, and MIP-1β (27-29). Other HIV-1 -specific immune responses have also been observed in HEPS cohorts such as IgA in mucosa (30) (31;32)and serum (33), and serum antibodies to HLA molecules (34;35) and CD4 (36). The contribution of these mechanisms to resistance to HIV-1 infection remains unclear.

As discussed above, published studies thus far have detected CTL in the blood of approximately 30-60% of HEPS individuals examined. The fact that HIV-1-specific $CD8^+$ immune responses are not detected in all HEPS tested suggests that other mechanisms could be involved in this protection, that the current assays used to detect the $CD8^+$ immune response lack the sensitivity to detect these responses, or that only 30-60% of HEPS are actually exposed to HIV-1 . One of the problems that exists in current assays systems is that there is a significant lack of defined CTL epitopes in individuals exposed to non-clade B strains of HIV-1 (37). Furthermore, it is likely that epitopes previously characterized in seropositive individuals are not necessarily going to be the same as those recognized by HEPS. For this reason, an important goal of the HPTN immunology core group is to enroll potential subjects identified as HEPS and ultimately test specimens for HIV-1 -specific $CD8^+$ mediated immune responses using clade-specific antigens and the most current and sensitive technologies available.

1.2 Rationale

The rationale for this study is based on several premises:

- The need to understand the immunological, viral and host factors related to the resistance to HIV-1 acquisition, by high-risk individuals, for the design and evaluation of HIV-1 prevention strategies for the HPTN.
- The unique opportunity to investigate the immunological, viral and host factors related to resistance to HIV-1 infection in Pune.
- The opportunity, through this laboratory protocol, to assess the feasibility of identifying, enrolling and retaining exposed partners and their highly infectious partners with recent HIV-1 infection, in a unique prospective cohort of discordant couples for future HPTN clinical trials.

The overall aim of this study is to investigate the immunologic, virologic and genetic factors that impact upon acquisition of or protection from HIV-1 infection. These studies would provide data that would be critical for designing prevention strategies involving the use of antiretrovirals, microbicides and vaccines.

An equally important goal of this study is to begin to transfer the technology of virologic and immunologic assays to the NARI HPTU, so that these measurements can be made on-site, instead of being shipped to and performed at a central location. Currently, novel immunologic assays, such as the ELISPOT and a flow cytometric assay for measuring intracellular cytokine levels are being developed and standardized for use on-site in large, multi-site clinical trials. The HPTN Central Laboratory (HCL) will coordinate the development of consensus protocols and transfer of the technology for immunologic assays to NARI. This proposal will be coordinated with the 034A HPTN Early Infection Laboratory Sub-study. The first step in performing these kinds of tests in a consistent, standardized way is to establish a well- organized, accessible repository of PBMC and plasma samples on-site. The goal of this protocol is to outline procedures to create such a repository for future studies to be carried out on-site when consensus protocols have been developed and implemented. This sub-study will also develop quality control measures to assure consistent sample collection and processing at the NARI HPTU site in preparation of future studies to be developed by the HPTN.

The HPTN will encompass the largest network to date of cohorts of acutely infected individuals and their uninfected partners from countries around the world that continue to have a significant incident of HIV-1 infection. These cohorts will allow for the characterization of distinct viral subtypes and of the immune response to these different subtypes of HIV-1 in individuals with genetically diverse backgrounds (eg HLA, co-receptor genotype, etc), as well as a variety of factors that may impact the primary immune response to HIV-1 (eg. micronutrient deficiency, co-infections, viral load). Understanding the significance of viral characteristics and mechanisms and their relationship to potential immune correlates of protection for HIV-1 , such as CTL recognition, is a primary goal of the HPTN. In addition, this protocol would provide standardization of specimen collection for additional HPTN protocol specific studies in the future.

2.0 STUDY OBJECTIVES AND DESIGN

2.1 Primary Objectives

- To determine if the detection of an HIV-1 -specific immune response in exposed uninfected individuals correlates with risk of subsequent HIV-1 infection.

- To determine if immunologic genetic characteristics of exposed uninfected individuals correlates with risk of subsequent HIV-1 infection.

2.2 Secondary Objectives:

- To determine if the detection of an HIV-1 -specific immune response in exposed uninfected individuals correlates with the HIV-1 viral set point in individuals that subsequently become HIV-1 infected.

- To compare the epitope specificity of the HIV-1 -specific immune response of exposed-uninfected individuals with that of their recently infected partners.

- To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples.

2.3 Study Design:

This is a prospective cohort study to be conducted at NARI. NARI will implement the study in accordance with this protocol, however additional studies and collaborations may be incorporated into this study with approval from the Executive Committee. NARI will document study data using standard data collection forms to be submitted to the HPTN Statistical and Data Management Center (SDMC).

An estimated 10-15 individuals/year who qualify as HIV-1 -exposed uninfected are expected to be enrolled in this study. To maximize efficiency and to utilize established systems for screening and follow-up, individuals who themselves are HIV-1 negative, but who have recently infected partners will be recruited from the ongoing 034 HPTN trial. Enrollment will be offered to all participants in HPTN protocols who fulfill the enrollment inclusion/exclusion criteria. Individuals will necessarily have had a previously documented negative HIV-1 test that will be confirmed by HIV-1 RNA PCR. Appendix A (Table 1) reflects the clinical and laboratory specimens that will be collected at each visit. In this protocol, only samples from exposed-uninfected individuals will be collected. Samples from seropositive and seronegative individuals enrolled in the 034AHPTN Early HIV-1 Infection Study Laboroatory Sub-study will serve as comparative controls for the assays performed in the exposed-uninfected study.

3.0 Study Population:

3.1 Inclusion Criteria for HEPS Subjects

Eligible subjects would include individuals from the following population:

- HIV-1 uninfected partners of those enrolled in the 034A HPTN Early HIV-1 Infection Laboratory Sub-study.

All of the following must be fulfilled:

- Willing and able to provide informed consent
- > 18 years of age
- Willing to permit phlebotomy of four tubes of blood every three months for 24 months

3.2 Exclusion Criteria

- An obvious psychological/psychiatric disorder that would invalidate the informed consent process or otherwise contraindicates participation in the study.
- HIV-1 infected
- Hemoglobin level < 7mgs/dl

3.3 Conditions for Participant Withdrawal

A participant may be discontinued early from the study for any of the following reasons:

- Participant withdraws consent/decides that s/he does not want to continue in the study.

- A clinician or the site PI decides that continued participation is not warranted or advisable due to health reasons or other reasons related to the well-being of the participant, including an obvious psychological/psychiatric disorder that would invalidate the informed consent process, or otherwise contraindicate participation in the study.

- Any other condition that in the opinion of the study site Principal Investigator will interfere with achieving the study objectives. The decision to withdraw the participant should be done in consultation with the Protocol Co-Chairs and HPTU Core Protocol Team.

Reasons for discontinuation from the study will be recorded on the appropriate case report form.

4.0 STUDY PROCEDURES

This section refers to laboratory procedures that will be used to evaluate outcomes for each of the primary objectives. The following procedures will be used to accomplish the primary objectives listed below.

4.1 Screening and Enrollment

Individuals who are identified as the sexual partner of participants enrolled in the 034 HPTN Early HIV-1 Infection Laboratory Sub-study will be eligible for enrollment in this protocol. Participants in the Early HIV-1 Infection study will be asked to provide consent to inform their partners of their recent HIV-1 infection. The partners who are then referred to the HPTU clinics for HIV-1 screening, by the participants in the HPTN Early HIV-1 Infection study, will be provided counseling and offered HIV-1 screening testing according to procedures at the NARI site, following informed consent. These exposed partners of recently HIV-1 infected individuals will be subsequently defined as either A) HIV-1 antibody positive, B) recently HIV-1 infected or C) HIV-1 -exposed, but uninfected (laboratory definitions of these three categories provided below). HIV-1 -antibody positive partners of recently HIV-1 infected subjects will be counseled and referred to care and support services available. Recently HIV-1 infected partners of recently infected subjects would themselves be eligible for enrollment in the companion HPTN Early HIV-1 Infection Study. HIV-1 -exposed, but uninfected partners of HIV-1 infected participants in the HPTN Early HIV-1 Infection Study would be eligible for enrollment in this HPTN protocol.

The schedule of visits and procedures is presented in Appendix A (Table 1). At enrollment, informed consent will be obtained and participants will be counseled appropriately for their HIV-1 status. Eligibility will be confirmed and locator information will be obtained. Demographic information will be obtained. A physical exam will be completed. If symptoms of a sexually transmitted disease are present at enrollment or during follow-up, diagnosis and referral for treatment will be provided as locally appropriate. Specimens will be collected, processed and stored or shipped according to the protocols outlined in the site-specific study manual. The next appointment will be scheduled and the appropriate data collection forms will be completed.

To maximize efficiency, established systems for screening and follow-up at each site will be utilized to the extent possible, including the use of home visitors, modest incentives, and tracking procedures. If a participant misses a scheduled study visit, the study site staff will try to establish communication with the participant through all possible means using the contact information provided by the volunteer. The need to adhere to the visit schedule will be emphasized at each contact. Participants may be compensated for meals, time away from work and for transportation to the study clinic as appropriate on a site-by-site basis.

At all visits (enrollment and follow-up) whole anticoagulated blood will be obtained using four 10 ml purple-top EDTA tubes. Blood should be processed within 12 hours of collection. NARI will follow specific protocols for processing and storage of PBMC and plasma.

Individuals with acute HIV-1 infection are highly infectious to their sexual contacts and with whom they may share needles. The ethical concerns surrounding this issue are important,

complex and will require comprehensive review and discussion. Of particular importance are the ethical issues concerning contact tracing, protecting the confidentiality and human rights of the participants and their sexual contact, as well as access to the best available local care for HIV-1 infection. These issues are important for all HIV-1 prevention studies for which HIV-1 infection (i.e. HIV-1 antibody seroconversion) is the primary or secondary endpoint. However, they are particularly important in the setting of acute HIV-1 infection, due to the increased risk of secondary transmission. Participants enrolled in this study will be eligible because of their exposure to partners with acute HIV-1 infection. Thus they will be strongly counseled to reduce their risk of subsequent sexual and needle exposure. It is therefore critical that the Pune site is committed to providing the best available risk reduction counseling for study participants throughout their participation in this protocol. If counseling of participants in this protocol is 100% effective, then none of the participants would subsequently be exposed to HIV-1 infection. Although this should be the goal of counseling for all of the study participants, this protocol is proposed with the recognition that a 100% reduction of risk is unlikely.

4.2 Follow-up

At each follow-up visit, the participant's identity and ID number will be verified. A physical exam and questionnaire will be completed. Specimens will be collected, processed and stored/shipped according the study-specific procedures manual (Appendix A). The next appointment will be scheduled (if required) and the appropriate data collection forms will be completed.

4.3 Laboratory Procedures

The laboratory procedures for this protocol are similar to what is proposed for the companion HPTN Early HIV-1 Infection Protocol. The training, technology transfer and quality control activities for these two protocols will be coordinated by the HCL.

4.3.1 Diagnosis of HIV-1 Infection:

NARI will utilize their own site-specific criteria for identifying individuals with HIV-1 infection. NARI will utilize commercially available HIV-1 antibody assay kits for HIV-1 screening. To be documented as HIV-1 antibody positive for the purposes of this protocol, eligible subjects must have a current positive HIV-1 antibody test and a negative HIV-1 antibody test result within the prior 6 months. A positive antibody test would be defined as either A) HIV-1 + by two different rapid tests B) HIV+ by two different serum ELISA tests C) HIV+ by one rapid AND one serum ELISA test or D) HIV+ by either a rapid test OR an ELISA AND by one HIV-1 western blot. As outlined above, individuals who are NOT HIV-1 antibody positive, based upon any of the above four criteria, will be eligible for enrollment in this protocol. In addition to identification of recent antibody seroconvertors, sites may utilize their own procedures for screening antibody negative specimens using either commercially available HIV-1 p24 Ag assays or HIV-1 PCR assays (see below) to determine eligibility for participation in this protocol.

4.3.2 HIV-1 PCR and Viral Load Testing:

The Pune site will be expected to perform HIV-1 PCR and viral load (VL) measurements for studies outlined in this protocol, as well as other potential HPTN protocols such as preparedness studies, perinatal studies, and ART protocols requiring VL measurements. The HCL will recommend the use of the Roche Amplicor HIV-1 Monitor™ Test version 1.5 because of its increased sensitivity and ability to detect non Clade B subtypes of HIV-1 (38). This assay would be recommended for sites interested in screening HIV-1 antibody negative specimens for identification of HIV-1 PCR+ subjects in the "window" period for enrollment in the companion HPTN Early HIV-1 Infection Study. This same assay would be utilized on-site for all VL assays for study participants. The HCL virology core will provide training for this HIV-1 PCR assay. This assay contains quality control material in the form of negative, low and high standards that will be used with each assay run. There is also an internal standard control that is assayed with each specimen. Sites will also be required to participate in QA proficiency testing on a quarterly or semi-annual basis through a DAIDS-sponsored Virology Quality Assurance (VQA) program already in place in the US. More detailed information on the viral load assay is provided in Appendix B.

4.3.3 Specimen Processing and Storage

The HCL immunology group will initially begin training NARI lab personnel for specimen collection and storage (if needed). This training can be performed using seronegative volunteer samples, to avoid using more limited seropositive samples. Therefore, sites will be expected to recruit 20 seronegative volunteers at the on-set of the study, and then up to 10 seronegative volunteers for each year of the study.

The negative control samples will be used for:

- Quality controls for freeze/thaw and viability protocols
- HLA screening for population genetics
- Negative controls in immunologic assays

All HPTUs must meet or exceed guidelines that will be outlined in the study-specific procedures manual for cell preparation and preservation quality (Appendix D). While it is clearly optimal to use freshly isolated PBMC for real-time assays, it is likely that in this study, and in future larger HIV-1 prevention and vaccine studies, the use of frozen specimens will be necessary for batch testing. It has been previously shown that cryopreserved PBMC can be used for detection of cellular immune responses in HIV-1 infected volunteers (39;40). NARI will be required to periodically perform quality control analysis of frozen cells by thawing single aliquots of cells from negative controls to assess cell number and viability (detailed in study-specific procedures manual). For example, it would be expected a PBMC recovery 50% or greater of the total number of PBMC stored would be achieved, and that the viability of thawed cells would be approximately 90%. This number is based on previously observed recoveries from cryopreserved PBMC from HIV-1 infected individuals (41;42) Additionally, sites with flow cytometry capacities will be able to do more sophisticated measurements of cell preservation by testing for cell surface marker integrity. It has been previously shown that, cryopreservation of

cells does not significantly alter the cell surface expression of most lymphocyte markers studied (43).

4.3.4 HLA typing

PBMC from HIV-1 uninfected individuals will also be used for HLA typing to be performed in collaboration with Dr. Thomas Williams' Laboratory at The University of New Mexico Health Science Center Albuquerque, New Mexico. NARI would be required to ship a single frozen vial of PBMC to this laboratory for HLA typing. There is increasing evidence that HLA type influences the course of a number of diseases such as HIV-1 and malaria (44;45). Furthermore, identification of CTL epitope specificities using HLA types for modeling binding motifs has become a standard practice in cellular immunology. Therefore, characterizing the HLA types of the population in Pune will be important in understanding risk of HIV-1 infection and the immune response subsequent to infection.

4.3.5 ELISPOT Assays

As mentioned above, NARI will be evaluated for their ability to collect and store blood specimens. Once the laboratory is able to adhere to the quality control requirements outlined above and in the site-specific study manual (Appendix C), they will be encouraged to participate training workshops at Johns Hopkins University as well as on-site and regionalized training. Training will be provided for participation in future virologic and immunologic studies of acute seroconvertors including measurements of T cell function through ELISPOT and flow cytometry analysis (where available). If procedural problems or inconsistencies occur in QC procedures, individual sites will be responsible for internal problem solving, however, communication with the HCL group will be encouraged. Personnel from the HCL will periodically visit the NARI site for technical support and site evaluation.

NARI will identify at lease two key personnel on site as responsible for supervision and implementation of the study protocols. A site PI should be one of the site contacts, and the other should be a key laboratory scientist directly involved in the processing and laboratory testing of specimens. Representatives of the participating site would be invited to join the Protocol Team to contribute to the design of the subsequent immunologic and virologic studies to be undertaken with the specimens in the site repositories. The HPTN Immunology Group will also invite and encourage participation and collaboration with investigators outside of the HPTN for future studies.

Measurement of HIV-1 -specific T lymphocyte responses to HIV-1 infection in exposed, uninfected participants in this protocol will require a highly sensitive assay. There are a number of available assays to be utilized to address the specific research objectives of this protocol, including ELISPOT, tetramer and intracellular cytokine/flow cytometric assays. At this time, the ELISPOT appears to be the assay that could be most efficiently, consistently and reliably initiated in participating HPTU laboratories. However, the HCL will continue to evaluate the available assays and will enlist the expertise of investigators within and without the HPTN and consider utilizing other assays in the future.

Therefore, NARI site laboratory personnel will initially trained to use the ELISPOT assay to measure the cellular immune response in HIV-1 uninfected individuals exposed to HIV-1. This assay has been used previously to detect the $CD8^+$-mediated immune responses in HIV-1 infected individuals (46-51). The ELISPOT was selected for use at HPTU most sites rather than the conventional CTL assay for several reasons. While the CTL assay is the "gold standard" for measuring $CD8^+$ function (*in vitro* cell killing) in HIV-1 infected individuals, it is extremely laborious and difficult to perform. It requires a long (14 day) *in vitro* stimulation period and the use of sodium ^{51}chromate. The CTL assay requires more than $20x10^6$ cells/assay, and is not considered to be a quantitative measurement of antigen-specific T cell frequencies. Recent studies have shown that the CTL assay and the ELISPOT assay are comparable in detection of $CD8^+$-mediated immune response in seropositive individuals (M. Altfeld, G. Ferrari, personal communications). Our laboratory as well as others has shown that the ELISPOT assay can be performed using cryopreserved PBMC. The ELISPOT assay is fairly simple to perform and requires only a brief, overnight *in vitro* stimulation period. It requires a fraction of the cells necessary for a comparable CTL assay. For a more comprehensive discussion of cellular assays currently in use for clinical immunology assessments, please refer to current reviews by Hickling and Whiteside (51;52).

The ELISPOT assay is an alternative functional assay that can measure $CD8^+$ T cell function. The read-out of the ELISPOT assay is secretion of IFN-γ from HIV-1 -specific T cells. Secreted IFN-γ is captured onto a membrane-coated ELISA plate by an IFN-γ-specific monoclonal antibody. It is then detected using a second biotinylated IFN-γ-specific monoclonal antibody, and developed using standard ELISA reagents. The number of spots counted in a particular sample well corresponds to the number of HIV-1 -specific T cells in the well/total number of cells added. Therefore, the relative frequency of cells that are specific for a particular antigen can be determined. This assay can also measure a positive and negative response for each sample tested. The positive control consists of the addition of PHA to replicates of PBMC samples. A majority of $CD3^+$ lymphocytes will secrete IFN-γ in response to this mitogen. The negative controls for this assay are mock-stimulated replicates in which no stimulus is added. This allows for the assessment of background levels of IFN-γ secretion. For purposes of quality assurance, the HCL will create a panel of frozen PBMC to serve as positive and negative control samples. These cells will be sent to the NARI site to test in a blinded fashion in the ELISPOT assay using a standard set of reagents.

An important issue for the ELISPOT assay is the choice of HIV-1 -antigens to use for the *in vitro* stimulation. The use of overlapping pools of HIV-1 gag peptides for *in vitro* stimulation has been shown to be an effective means of characterizing the gag-specific immune response to HIV-1 in acute and chronic infection (46-51). One complicating factor that will need to be addressed is whether to use sequence-specific HIV-1 antigens for the *in vitro* stimulation. Designing peptides that match the predominant HIV-1 strains to which study participants may be exposed at a particular site would be extremely difficult and expensive. Furthermore, because there is limited data on the importance of customizing overlapping peptide sequences to match subtypes of HIV-1 represented at each site, it is not clear that this would outweigh the substantial cost of synthesizing multiple sets of peptides. Therefore, the HCL will attempt to provide reagents that are based on the best information currently available on viral subtype amino acid sequences. These sequences will be obtained from sequencing data from the sites themselves, sequences

published in the current literature, and the compendium of sequence data from the HIV-1 molecular database at the Los Alamos National Laboratory (53).

Based upon the data from prior studies and an initial assessment of the logistical limitations of initiating this type study at multiple HPTU, this proposal will initially focus on measurement of HIV-1 gag-specific ELISPOT responses utilizing a matrix format of pools of overlapping HIV-1 gag peptides(54-57). NARI will initially be provided with a set of HIV-1 subtype C gag peptides. Based upon this initial analysis of the gag-specific responses and upon data from other ongoing studies, we propose to consider expanding this analysis to include assessment of other HIV-1 antigens, including env, pol, nef and tat. The details of the proposed consensus ELISPOT assay with gag peptides are provided in Appendix C. ELISPOT measurements will be made as early and frequently as possible after initial identification of infection. The visit schedule is outlined in Appendix A. The need for these frequent blood draws is comes from data that suggests that the epitope recognition seen in exposed uninfected individuals may be transient and variable.

Based upon the analysis of the ELISPOT data, a decision will be made by a consensus of the protocol team, which includes NARI site investigators and investigators outside of the HPTN, about expanding the analyses to other immunological assays (e.g. tetramer, intracellular flow cytometric assays, co-receptor genetic analysis), other antigens (e.g. nef, pol, env, tat) and other critical scientific questions. New information is continuously being generated that impacts our understanding of events critical to HIV-1 transmission and pathogenesis. To optimize the relevance of this study to the HPTN, the protocol team must include outside expertise and must stay informed about the latest information that might suggest modifications in the specifics of the laboratory assays utilized for this protocol. This protocol must also facilitate and be coordinated with ongoing, related laboratory studies at participating HPTUs, as well as with outside collaborators. The science in this field moves quickly and this laboratory protocol must remain dynamic, flexible, informed and collaborative to optimize this important scientific opportunity.

5.0 Evaluation of Outcomes

5.1 Primary Outcomes

Following the successful implementation of immunologic (ELISPOT) and virologic (viral load measurements) assays at NARI (See quality control and training details above), this project will collect data to address the two stated primary objectives.

5.1.1.Primary Objectives (a).

To determine if the detection of an HIV-1 -specific immune response in exposed uninfected individuals, correlates with risk of subsequent HIV-1 infection.

The initial research hypothesis for this objective, based upon a few prior studies of HIV-1 exposed, uninfected individuals, is that exposed uninfected individuals with CD8-mediated recognition of HIV-1 -gag epitopes will demonstrate a lower risk of subsequent HIV-1 infection, compared with exposed individuals that do not recognize HIV-1 gag epitopes. A further hypothesis is that this correlation will be demonstrated in exposed uninfected individuals, who are exposed to recently infected individuals infected with different strains of HIV-1. The primary outcome measures for this analysis will be

subsequent HIV-1 infection within the first 2 years of follow-up, and demonstration of an HIV-1 gag-specific response measured in an ELISPOT assay. Based upon this initial analysis of gag-responses and upon data from other ongoing studies, we propose to consider expanding this ELISPOT analysis to include assessment of recognition of other HIV-1 antigens, including env, nef and tat.

5.1.2.Primary Objectives (b).
To determine if immunologic genetic characteristics of exposed uninfected individuals correlates with risk of subsequent HIV-1 infection, across diverse populations.

The initial research hypothesis for this objective, based upon prior studies in a few countries demonstrating that certain HLA class I alleles may be associated with a lower risk of HIV-1 infection, is that there are specific host genetic characteristics that will be associated with a lower risk of HIV-1 infection in exposed individuals from diverse populations infected with different strains of HIV-1. The primary outcome measure for this analysis will be subsequent HIV-1 infection, defined as above, and HLA class I allelic genotype. Based upon this initial analysis of the HIV-1 class I alleles and upon data from other ongoing studies, we propose to consider expanding this analysis to include assessment of HLA Class II alleles and co-receptor genotyping.

5.1.3 Primary Endpoints

(a) HIV-1 antibody seroconversion
(b) ELISPOT measurement of HIV-1 peptide-specific T lymphocytes
(c) HLA-Class I MHC alleles

5.2 Secondary Outcomes

5.2.1 Secondary Objectives (a)
To determine if the detection of an HIV-1 -specific immune response in exposed uninfected individuals, correlates with the HIV-1 viral set point in individuals that subsequently become HIV-1 infected.

For HIV-1 exposed subjects that subsequently seroconvert, viral load measurements will be taken to determine if subjects with detectable ELISPOT responses to the gag protein had lower viral set point than those HIV-1 exposed subjects that had no detectable immune response to the gag protein.

5.2.2 Secondary Objectives (b)
To compare the epitope specificity of the HIV-1 -specific immune response of exposed-uninfected individuals with that of their recently infected partners.

In cases where positive responses are detected in the initial screen of the ELISPOT assay, additional assays will be performed on stored specimens to determine the phenotype of the responding cell, epitope specificity and MHC restriction. ELISPOT responses to HIV-1 gag peptides in recently infected subjects and their exposed uninfected partners will be compared to see in the same sub-set of epitopes is recognized in those discordant couples that share HLA alleles.

5.2.3 Secondary Objectives (c)

To assess the feasibility of establishing a prospective cohort of recently infected individuals and their primary sexual partners for participation in HPTN HIV-1 prevention trials of discordant couples.

This laboratory study will provide an opportunity to assess the feasibility of establishing and following cohorts of recently infected individuals and their sexual partners for future HPTN trials. Therefore enrollment and retention rates will be enumerated and evaluated for the Pune site.

5.2.4 Secondary Endpoints

(a) Median HIV-1 viral load between 6 and 12 months after estimated time of infection, for participants that HIV-1 seroconvert.
(b) HLA restriction and minimal peptide sequence of responding T lymphocytes from exposed uninfected participants.
(c) Enrollment and retention of study participants and their acutely infected partners enrolled in the 034A HPTN Early Infection Laboratory Sub-study.

6.0. Data Collection

6.1 Data Forms

Data will be collected on standardized forms to be outlined in the site-specific procedures manual. An appropriate system for data transfer from the sites to the HPTN Core Laboratory and for central data management, quality assurance and analysis will be developed and utilized. A data and care report forms flow chart will be included in the study specific procedures manual along with procedures for data collection, transfer, and management.

6.2 Record Storage and Archive

The study site principal investigator and laboratory personnel will maintain all source documents used to complete case report forms including laboratory requisitions and reports, documentation of referrals, and progress notes. Specimens will be tracked using the Laboratory Data Management System (LDMS). All data collection forms and source records must be kept in locked files in a secure area. All study documents with participant identifying information including locator information and informed consent forms must be kept in locked files in a secure area apart from all other study documents. Study records must be available at all times for review by FHI, the study sponsor (NIAID) or their agents.

6.3 Statistical Considerations and Sample Size Calculations

Based upon assumptions outlined below for each of the two primary objectives, the target samples size for this study is a minimum of 20 subjects who are HIV-1 -exposed uninfected individuals with recently infected partners. For both primary objectives, frequency of HIV-1 infection will be an outcome measure. Patients will be divided into an infected and uninfected

cohort and compared with respect to the presence or absence of any prior detectable immune response as measured by Elispot.

6.3.1 Primary Objectives (a).
Gag-specific ELISPOT Response vs. subsequent HIV-1 Infection.

For the purposes of comparing ELISPOT response to incidence of infections, gag-specific ELISPOT responses will be scored as positive or negative. As outlined in Appendix C, all experiments will be performed in duplicate wells. The IFN-γ-expressing spots will be calculated as the number of spots per 10^5 stimulated PBMCs. Results will be expressed as net peptide-specific IFN-γ spots / 10^5 PBMC. A positive peptide-specific response (responders) will be defined as a mean of greater than 5 spots above the mean spots of the mock-stimulated PBMC. Study participants will be expected to have multiple ELISPOT assays within the first 12 months of follow-up (See Appendix A). For the purpose of the sample size calculation, our initial analysis will compare HIV-infection with a positive response to any gag peptide, at any time point within 12 months since enrollment. Separate and similar analyses will be performed for recognition of HIV-1 subtype B gag and non-B gag peptides. Assuming a 20% seroconversion rate among the exposed uninfected cohort resulting in 20 acutely infected patients, we would have 80% power to detect at least a 25% difference in the proportion of individuals who elicit a gag immune response between the two groups (infected and uninfected).

Difference in proportion of patients with gag response	Total sample size (unequal arms assuming 20% seroconversion rate)
20%	238
25%	138
30%	86

6.3.2 Correlation of HLA allelic expression with risk of subsequent infection.

The proportion of individuals with certain HLA Class I alleles will be compared between infected and uninfected individuals. Using HLA A2 as an example (known to exhibit good immune response to gag), we posit that a higher percentage of this allele will be found in the uninfected cohort. Using a similar calculation to 6.3.1, we will have 80% power to detect at least a 25% difference in allelic frequency between the two groups.

7.0 Human Subjects Considerations

7.1 IRB Review

This protocol, the proposed informed consent documents and any subsequent modifications will require review and approval by a number of Institutional Review Boards or Ethics Committees, responsible for oversight of the HPTN activities. The activities of this protocol will be covered by an approved HPTN OPRR International Cooperative Project Assurance (ICPA) for each participating site, required for participation in an OPRR-recognized HPTN Cooperative Protocol Research Program (CPRP). It will be the responsibility of participating site investigators to

obtain and document all required approvals for this study. Specifically site participation in this protocol will require documentation of ethical review and approval from the following:

- International HPTUs: Local institutional IRB and IRB of collaborating US institution, when applicable.

- Domestic US HPTUs: Local institutional IRB

7.2 Confidentiality

All local study records will be kept in locked file cabinets in areas with limited access; databases will be secured with password-protected access systems. A coded ID number will identify all study data and specimens only, and records containing participant names or other personal identifiers, such as locator forms and informed consent documents will be stored separately from coded ID records.

7.3 Benefits

Support for HIV-1 Prevention
During the study, the participant will receive the most current information and counseling about how to avoid acquiring or transmitting HIV-1 to partners. Studying immune responses in early HIV-1 infection will provide important knowledge concerning strategies for prevention and vaccine development and evaluation. In light of the high risk of secondary HIV-1 transmission to sexual partners from individuals with recent HIV-1 infection, an important effort will be made to encourage participants to refer their partners for HIV-1 screening and counseling.

Access to Care
The study staff will refer participants for STD treatment and other services as needed according to standard procedures at each site. Each participating HPTU site will be responsible for assuring compliance with their own ethical guidelines for providing the best locally available clinical care to study participants.

7.4 Risks

Health Risks
Study participants may feel discomfort associated with phlebotomy, including pain, bruising and/or in very rare instances, local infection.

Psychosocial Risks
Periodic testing of viral and immunologic indices may increase participant's anxiety level concerning their health and risk of HIV-1 infection.

7.5 Study Discontinuation

The study may be discontinued at anytime by FHI, the HPTN, NIAID or by the government and host-institution of the country in which the study is being conducted.

7.6 Incentives

Participants may be provided a small incentive in the form of a transport allowance and free access to medical services for their participation in this study, as determined by individual sites based on local standards of practice and in accordance with ongoing studies from which the participants will be identified. The details of these incentives will vary from site to site, but must be included in the local IRB approved consent form for each participating HPTU.

8.0 Laboratory Specimens and Biohazard Containment

As the transmission of HIV-1 and other blood-borne pathogens can occur through contact with contaminated needles, blood, and blood products, appropriate blood and secretion precautions will be employed by all personnel when drawing of blood and shipping and handling of all specimens for this study, as currently recommended by the Centers for Disease Control and Prevention.

9.0 Administrative Procedures

9.1 Study Coordination

Close cooperation between the protocols team and site investigators, protocol coordinator, data managers and biostatisticians will be necessary; study progress will be evaluated by the study team on a regular basis via conference calls. The sites will employ a common study specific procedures manual (with site-specific sections as appropriate). This manual will outline procedures for enrollment, follow-up, data and forms processing and other study operations.

A common study laboratory manual will be followed to standardize specimen collection, preparation, testing, processing, and shipping. For laboratory procedures and assays (i.e., cell separations, freezing of samples, flow cytometry, etc.) that can be performed by the local site laboratory, or the HCL. Other assays will be performed at the HCL or in the laboratories of non-HPTN participants/collaborators.

Every effort will be made to provide training and technology transfer to each of the sites to increase their overall capabilities. There will also be a strong commitment to rapid bi-directional exchange of test results and other data to facilitate the collaborative interpretation and timely publication of HPTN findings.

9.2 Study Monitoring

The HCL will conduct site visits to monitor adherence to human subjects and other applicable regulations, adherence to the study protocol and procedure manuals, and the quality of the data collected at the study sites. The study site key investigators will allow the inspection of study documents (e.g., consent forms, process data collection forms, questionnaires) for confirmation of the study data.

9.3 Protocol Compliance

This study will be conducted in full compliance with the protocol. The protocol will not be amended without prior written approval by the Protocol Chairs and DAIDS Medical Officer. Protocol amendments requiring IRB approval must be approved by the IRB prior to implementing any amended procedures.

9.4 Investigator Records

All records must be retained on-site throughout the study's period of performance. The HPTN will provide each site with written instructions for long-term record storage at the completion of the period of performance.

Study records include the following:

- Administrative files, including initiation documents and all reports and correspondence relating to the study.
- Records for each participant, including informed consent forms, locator forms, data collection forms, and source documents

9.5 Use of Information and Publications

The policies that govern specimen ownership, intellectual property rights, and the publication/authorship of research will be derived from the WHO policy document concerning these issues. Virus isolates and any remaining serum/plasma/cell samples, which are not consumed during the specified testing, will be retained in a specimen archive at the local site, the HPTN laboratory, or the NIAID Repository contractor (BRInc.) in Bethesda MD. These samples will be available to any responsible investigator for further studies, pending review of the request by the site that submitted the sample and the HPTN Steering Group or the appropriate oversight committee following HPTN.

Publication of the results of this study will be governed by policies of DAIDS and the HPTN. Any presentation, abstract, or manuscript will be made available by the investigators to DAIDS and the HPTN manuscript review committee for review and comment prior to submission.

10.0 REFERENCE LIST

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Ref Type: Internet Communication

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APPENDIX A:

Table 1. Scheduled visits for exposed-uninfected individuals

	Screening	Enrollment												
Month	Pre	0	3	6	9	12	15	18	21	24	27	30	33	36
Study Visit	01	02	03	04	05	06	07	08	09	10	11	12	13	14
Patient ID	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Physical Exam	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Questionnaire	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Assays:														
HIV-1 Test[1,]	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HIV-1 RNA PCR[2]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]	[X]
Sample Storage:														
Plasma[3]	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PBMC		X	X	X	X	X	X	X	X	X	X	X	X	X
HLA Typing[4]		X												
Total blood volume (ml)	10	40	40	40	40	40	40	40	40	40	40	40	40	40
Number of Tubes [5]	1	4	4	4	4	4	4	4	4	4	4	4	4	4

[1]HIV-1 screening will be done at each visit to confirm HIV-1 antibody negativity (screening assay will be site-specific).
[2]HIV-1 RNA PCR will only be performed upon confirmation of infection.
[3]Plasma will be stored for detuning assay, VL measurement.
[4]Specimen type to be determined.
[5]Tubes will be 10ml lavender top tubes containing EDTA.

Appendix B Viral Load Measurement

Procedure:
AMPLICOR HIV-1 MONITOR TEST Version 1.5—STANDARD METHOD

SPECIMEN:

Plasma

Type: Blood will be collected in EDTA tubes.

Serum specimens and specimens collected in **heparin** are unsuitable for this test.

Standard precautions will be observed for the collection, handling, transport, and processing of all specimens.

Handling Conditions:

Follow procedures outlined in the PBMC isolation protocol.

MATERIALS AND EQUIPMENT:

Materials:

AMPLICOR HIV-1 MONITOR test kit, v 1.5

(1.) Specimen Preparation Reagents
AMPLICOR HIV-1 MONITOR Lysis Reagent
AMPLICOR HIV-1 MONITOR Quantitation Standard
AMPLICOR HIV-1 MONITOR Specimen Diluent
Isopropanol
70% Ethanol (**not denatured**), v/v with deionized water

(2.) Control Reagents
Negative Plasma (NHP)
AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)
AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)
AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)
VQA Controls (For ACTG samples/studies)

(3.) Amplification Reagents
AMPLICOR HIV-1 MONITOR Master Mix
AMPLICOR HIV-1 MONITOR Manganese Solution

(4.) Detection Reagents
MONITOR Denaturation Solution
MONITOR Hybridization Buffer
AMPLICOR Avidin-HRP Conjugate
AMPLICOR Substrate A
AMPLICOR Substrate B
AMPLICOR Stop Reagent
AMPLICOR 10X Wash Concentrate
Distilled or deionized water
AMPLICOR HIV-1 MONITOR Microwell Plate

Reagents not included in kit

(1) 95% ethanol (freshly diluted to 70% with deionized water)

(2) Isopropanol, reagent grade

Equipment:

(1.) Perkin Elmer GeneAmp® PCR system 9600 or 2400 thermal cycler
(2.) Consumables: tubes (MicroAmp Reaction Tubes PE #N801-0533),
Caps (PEN801-0535)
Base(PE N801-0531)
Tray and retainer (PE N801-0536)
(3.) Aerosol resistant pipette tips capable of holding 50 to 1000 microliters to prevent specimen and amplicon contamination
(4.) Eppendorf repeater pipet and 1.25ml Combitip Reservoir
(5.) Pipettors, adjustable volume (20 – 200 µl), (50 µl), (200 µl) Pipettes should be within 3% of stated volume.
(6.) Impact Pipettor
(7.) Narrow tip, sterile transfer pipettes
(8.) Latex or nitrile gloves, **powder-free**
(9.) Cryovials with caps, sterile, 2ml capacity. Screw caps must be used to prevent specimen splashing and potential contamination No snap caps
(10.) Tube racks for cryovials
(11.) Microwell plate sealers
(12.) Disposable reagent reservoirs
(13.) Disposable plastic bags
(14.) Biological Safety Cabinet (BSC), Template Tamer Box, or equivalent
(15.) Microplate Washer capable of washing a 96-well plate with 350-450 ul at 30 second intervals
(16.) Microplate Reader with the following specifications: bandwidth = 10 $\pm$ 3 nm, absorbance range = 0 to a minimum of 2.00 when read at 450 angstroms, repeatability = 1% accuracy = 3% from 0 to 2.00 when read at 450 angstroms, drift <0.01 per hour
(17.) Centrifuge
(18.) Microcentrifuge max RCF 16,000 x g, min RCF 12,500 x g
(19.) Vortex mixer
(20.) Dry incubator 37°C($\pm$ 2°C)
(21.) Personal computer with spreadsheet software
(22.) Graduated cylinders, 100 to 1000 microliter capacities
(23.) Sterile bottles
(22.) Disposable pipettes, 5ml and 10ml
(23.) Absorbent backed paper
(24.) Disposable gown
(25.) Clorox or equivalent

WARNINGS AND PRECAUTIONS:

(1.) Do not pool reagents from different lots or from different bottles of the same lot.
(2.) Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
(3.) Do not use kit after expiration date.
(4.) Material Safety Data Sheets (MSDS) are available on request from Roche Response Center or your local Roche office.

(5.) Three work areas are required: 1) a Rnase free area, 2) a Pre-Amplification extraction area and 3) a Post Amplification area. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification Area. Pre-Amplification activities must begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Gloves must be worn in each area and removed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-Amplification supplies and equipment must be confined to the Post-Amplification Area at all times.

(6.) This kit contains a component (NHP) derived from human blood. The source material has been assayed by the US FDA approved tests and found non-reactive for the presence of Hepatitis B Surface Antigen and antibodies to HIV-1-1/2 and HCV. No known test methods can offer complete assurance that product derived from human blood will not transmit infectious agents. Therefore, NHP should be handled as if infectious.

(7.) HIV-1 QS, HIV-1 DIL, HIV-1 MMX, HIV-1 Mn^{2+}, HIV-1 low, and high positive control contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing materials down laboratory sinks, flush the drains with large amounts of water to prevent azide buildup.

(8.) Wear eye protection, laboratory coats and disposable gloves when handling HIV-1 LYS, HIV-1 MMX, Monitor Denaturation, Monitor HYB, AV-HRP, SUB A, SUB B, Working substrate mixed, and Stop solution. Avoid contact of these materials with the skin, eyes, or mucus membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

(9.) SUB B and Working Substrate contain dimethylformamide, which has been reported to be toxic in high oral doses and may be harmful to the unborn child. Skin contact, inhalation of fumes and ingestion should be avoided. If skin contact occurs, wash thoroughly with soap and water and seek medical advice immediately.

(10.) Do not allow HIV-1 LYS, which contains guanidine thiocynate, to contact bleach. This mixture can produce a highly toxic gas.

(11.) Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. Do not use snap cap tubes.

PREPARATION:

Specimen Preparation Reagents:

(1.) AMPLICOR HIV-1 MONITOR Lysis Reagent

A tris buffered solution containing guanidine thiocyanate, dithiothreitol and glycogen.
Store at 2 to 8°C until expiration date. Warm at 30-37°C to dissolve any salt crystals that form.

(2.) AMPLICOR HIV-1 MONITOR Quantitation Standard (QS)

A buffered solution containing Quantitation standard RNA, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(3.) Working Lysis Reagent

Add 100µl of the QS to one bottle of Lysis reagent. Mix thoroughly. The pink dye is used as a visual confirmation that the QS has been added to the lysis reagent.
Discard remaining QS. Store at room temperature and use within 4 hours.

(4.) AMPLICOR HIV-1 MONITOR Specimen Diluent

A buffered solution containing Tris-HCl, EDTA, poly rA RNA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(5.) Isopropanol(2,2 – proponal)

Store at room temperature in the flammable liquids cabinet.

(6.) 70% Ethanol (not denatured), v/v with deionized water

Dilute absolute ethyl alcohol with distilled, deionized water to a 70%. (Volume depends on the grade of ethanol – 95% to 100%). Store at ethanol in flammable liquids cabinet. Prepare fresh 70% daily.

Control Reagents:

(1.) Kit Controls

A. Negative Plasma (NHP)

Human plasma that has been found to be non-reactive by FDA licensed test for antibody to HCV, antibody to HIV-1/2, and HbsAg, containing ProClin 300. Store at 2 to 8°C until expiration date.

B. AMPLICOR HIV-1 MONITOR Negative Control (HIV-1 (-)C)

A buffered solution containing poly rA RNA, Tris-HCL, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

C. AMPLICOR HIV-1 MONITOR Low Positive Control (HIV-1 L(+)C)

A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

D. AMPLICOR HIV-1 MONITOR High Positive Control (HIV-1 H(+)C)

A buffered solution containing a non-infectious RNA transcript with HIV-1 sequence, poly rA RNA, Tris-HCl, EDTA, and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

Amplification Reagents

(1.) AMPLICOR HIV-1 MONITOR Master Mix

A bicine buffered solution containing <37% glycerol, potassium acetate, <0.07% dATP, dCTP, TTP, dUTP, biotinylated primers, <0.0004%r *Tth* Pol, <0.01% AmpErase and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(2.) AMPLICOR HIV-1 MONITOR Manganese Solution

A solution containing manganese acetate and 0.05% sodium azide. Store at 2 to 8°C until expiration date.

(3.) Working Master Mix

Add 100µl of AMPLICOR HIV-1 MONITOR Manganese solution to one tube of AMPLICOR HIV-1 MONITOR Master Mix. It is not necessary to measure the volume of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10 – 15 times or by mixing with a vortex for 3 – 5 seconds. The pink dye is used for visual confirmation that the Manganese solution has been added. Discard the remaining Manganese Solution. Working Master mix should be stored at 2 - 8°C and use within 4 hours.

Detection Reagents:

(1.) MONITOR Denaturation Solution

A solution of EDTA, 1.6% sodium hydroxide, and amaranth dye. Store at 2 to 25°C until expiration date.

(2.) MONITOR Hybridization Buffer

A sodium phosphate solution containing <0.2% solubilizer and <25% sodium thiocyanate. Store at 2 to 25°C until expiration date.

(3.) AMPLICOR Avidin-HRP Conjugate

An avidin-horseradish peroxidase conjugate in a buffered solution containing 1% ProClin 150™, emulsifier, bovine gamma globulin and 0.1% phenol. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first.

(4.) AMPLICOR Substrate A

A citrate solution containing 0.01% H_2O_2 and 0.1% ProClin 150. Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first. Do not expose to metals, oxidizing agents or direct light.

(5.) AMPLICOR Substrate B

Contains 0.1% 3,3',5,5'-tetramethylbenzidine in 40% dimethylformamide. May cause harm to an unborn child. Harmful by inhalation and contact with skin.
Store at 2 to 8°C until expiration date. Once opened these reagents are stable for 3 months or the expiration date, whichever comes first. Do not expose to metals, oxidizing agents or direct light.

(6.) Working Substrate Solution

For each microplate, mix 12ml of Substrate A with 3ml of Substrate B. Protect from light. Store at room temperature. Use within 3 hours. Do not expose to metals, oxidizing agents or direct light.

(7.) AMPLICOR Stop Reagent

Contains 4.9% sulfuric acid. Store at 2 to 25°C until expiration date.

(8.) AMPLICOR 10X Wash Concentrate

A sodium phosphate and sodium salt solution containing EDTA, <2% detergent and 0.5% ProClin 300. Store at 2 to 25°C until expiration date.

(9.) Wash Buffer 1X

Dissolve any precipitate by warming to room temperature. Add 100ml of 10X wash concentrate to 900ml of distilled, deionized water. Mix well. Store in a clean, closed container at 2 to 25°C for up to 2 weeks. Label with preparation and expiration dates.

(10.) AMPLICOR HIV-1 MONITOR Microwell Plate

An oligonulceotide probe coated microwell plate with twelve 8 well strips in one resealable pouch with desiccant, HIV-specific DNA probe (rows A-F), Quantitation Standard specific DNA probe (rows G-H). Store at 2 to 8°C in the foil pouch. The plate is stable in an unopened pouch until the expiration date. Once opened the plate is stable for 3 months or until expiration date, whichever comes first) as long as it is store in the resealable pouch.

CALIBRATION:

None

QUALITY CONTROL:

It is recommended that one replicate of each of the following controls: HIV-1 Monitor (-), (L+), and (H+) be included in each test run. As with any new laboratory procedure, new operators should consider the use of additional controls until such a time as high degree of confidence is reached in their ability to perform the test correctly.

All controls and patient specimens should yield OD values for the QS that meet the criteria described in the Results section, demonstrating that the specimen processing, reverse transcription, amplification, and detection steps were performed correctly. If any specimen has a QS OD value that does not meet the criteria described above, the result for that specimen is invalid, but the run is still acceptable. If any control has a QS OD value that does not meet the criteria described above, the entire run is invalid.

The expected range for each of the controls is specified for each lot of control and is provided with the kit. The copy number/ml should fall within the range indicated on the provided sheet. This range is to

be used until at least 10 values have been run and an in-laboratory range can be established. These ranges should be checked periodically and any trends notes. The negative control should yield a "not detected" result; i.e. all HIV OD values less than 0.200. If one of the positive control values falls outside of the 2 standard deviations, but within 3 standard deviations, the run can be accepted. When the assay is run the next time and same control is out, further investigation needs to be done. If both positive controls have values outside of 2 standard deviations but within 3 standard deviations, the results should be reviewed by the laboratory director for final approval. If one of the positive controls falls outside of 3 standard deviations while the other positive control is in, the results need to be reviewed, with the possibility of the range of values around the out of control to be repeated. If the next time the assay is run and the same control is out of range then further investigation is needed.

It is required that the results be reviewed by the supervisor, director, or designee before they are released.

TROUBLESHOOTING/MAINTENANCE:

Maintenance is to be performed on the plate washer, reader, and thermal cycler. The maintenance on the plate washes will vary depending on the model and brand but the system should be bleached at least monthly. The plate reader needs to be calibrated monthly. There is daily, weekly, monthly and semi-annual maintenance to be performed on the thermal cycler. Check the Perkin Elmer manual.

PROCEDURE-STEPWISE:

Preliminary Statements

All reagents should be at room temperature before using them.

Workflow in the laboratory should proceed in a uni-directional manner, beginning in the Reagent Preparation Area and moving to the Specimen Preparation Area and then to the Amplification/Detection Area.

Run Size (hospital patients): Each kit contains sufficient reagents for 2 -12 test batches, which may be performed separately or simultaneously. It is recommended that one replicate of the HIV-1 Monitor (-) control, (L+) control, and (H+) control be included in each run.

Workflow: The AMPLICOR HIV-1 MONITOR Test can be completed in one day or over two days. If the testing is to be done in a single day, follow the instructions in order. If the testing is to be completed in 2 days the procedure may be stopped after specimen preparation or after amplification. To perform the specimen processing on day one and amplification/detection on day two complete section B through step where you will freeze the samples at -20°C until amplification can occur. On day 2 begin with section A, thaw the processed specimens at room temperature and then continue with step in section B. To complete specimen preparation and amplification on day 1 and detection on day 2, perform sections a, b, c on day 1 and store the denatured amplicon at 2 to 8°C for up to one week. Continue with section D on day 2.

Reagent Preparation

(1.) Preparation working Master Mix by adding 100 microliters of Manganese Solution to one tube of Master Mix. Recap the Master Mix tube and mix well by inverting the tube 10-15 times (this is "working master mix"). The pink dye in the Manganese Solution is for visual confirmation that the

Manganese Solution has been added to the Master Mix. Leftover Manganese Solution should be discarded.

(2.) Place the appropriate number of PCR reaction tubes, 12 tubes to a row, into a MicroAmp sample tray and lock the tubes in position with the tube retainer.

(3.) Pipette 50 microliters of working master mix into each PCR tube using a micropipettor with an aerosol resistant tip. Discard leftover working master mix.

(4.) Place the microtube tray in a plastic zip-lock bag and store the tray at 2 to 8°C until the specimen preparation is completed. **Amplification must begin within 4 hours of the preparation of the working master mix**.

(5.) If performing the specimen preparation in the same area, remember to clean the area. Remove gloves and dispose of them properly. Remove lab coat and/or any other protective garments.

Specimen and Control Preparation

(1.) Prepare 70% ethanol, volumes depend on if using 95% ethanol or 90% ethanol.(for 12 tests, mix 11ml of 95% ethanol and 4ml of deionized water.

(2.) Prepare working Lysis Reagent as follows:

Warm the cold Lysis Reagent until the crystals have dissolved. Mix for at least ten seconds prior to use to ensure that it is thoroughly mixed.

Add 100ul QS. The lysis reagent will now be pink.

(3.) Label a 2.0ml screw cap microcentrifuge tube for each sample, including one tube for each kit control and/or standard.

(4.) Thaw plasma specimens and if used, standards to room temperature and vortex each tube for 3-5 seconds.

(5.) Briefly centrifuge each specimen tube in order to collect the sample into the base of the tube. Do not contaminate gloves while manipulating specimens.

(6.) Dispense 600µl of working Lysis Reagent into each labeled microcentrifuge tube. Check that the working lysis reagent is pink to confirm that the QS was added to the lysis reagent.

(7.) Add 200µl of plasma or standard to each appropriately labeled microcentrifuge tube containing the working Lysis Reagent. To the controls, add 200µl of NHP and 50µl of the controls to the microcentrifuge tube. Re-cap the tube and vigorously vortex for 3-5 seconds.

(8.) Incubate the tubes for 10 minutes at room temperature.

(9.) Remove the cap from each tube and add 800µl of 100% Isopropanol to each tube. Re-cap the tubes and vigorously vortex them for 3-5 seconds.

(10.) Place an orientation mark on each tube and place the tubes into the microcentrifuge with the orientation marks facing outward, so that the pellet will align with the orientation marks. Centrifuge the tubes at maximum speed (at least 12,500 x g) for 15 minutes at room temperature. Upon completion of the centrifugation carefully remove each tube from the rotor,

(11.) Beginning with the control tubes, carefully draw off the supernatant, without disturbing the pellet (which may not be visible), using a fine tip, disposable transfer pipette. **Remove as much liquid as possible without disturbing the pellet: slide the pipette down the inside of the tube along the side opposite the pellet while drawing off the liquid. Maintain a continuous negative pressure with the transfer pipette as you draw off the liquid**.

(12.) Add 1ml of 70% ethanol to each tube, re-cap, and vigorously vortex 3-5 seconds.

(13.) Place the tubes into the microcentrifuge with the orientation marks facing outward and centrifuge the sample at maximum speed for 5 minutes at room temperature.

(14.) Carefully remove the tubes from the centrifuge rotor and aspirate the supernatant as described as above. The pellet should be clearly visible at this time. **Remove as much of the supernatant as possible! (Do this twice). Residual ethanol can inhibit the amplification.**

(15.) Add 400µl of Specimen Diluent, re-cap, and vigorously vortex for at least 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

(16.) Amplify the processed specimens within 2 hours of preparation or store frozen at -20°C for up to one week.

(17.) Preparation for amplification: If previously frozen, warm extracted specimens to room temperature, vigorously vortex each tubes for at least 10 seconds.

(18.) Pipette 50µl of extracted specimens, controls to the appropriately designated reaction tubes which were previously prepared using a micropipettor with a plugged tip. Use a new tip for each specimen and control.

(19.) Securely cap each tube and using the MicroAmp capping tool seal the tubes.

(20.) Transfer the tray with sealed tubes containing the processed specimens and controls in working master mix to the Amplification/Detection area.

(21.) Decontaminate work area with a 1:10 dilution of bleach. Follow by cleaning area with propanol.

Reverse Transcription and Amplification

NOTE: Turn on the GeneAmp PCR System 9600 thermal cycler at least 30 minutes prior to beginning amplification.

(1.) Place the reaction tray into the thermal cycler sample block. **Make sure that the notch in the reaction tray is at the left of the block, and that the rim of the tray is seated in the channel around the block**.

(2.) Make certain that the cover knob is turned completely counterclockwise and slide the cover forward.

(3.) **Turn the cover knob clockwise until hand tight**.

(4.) Program the GeneAmp System 9600 thermal cycler as follows:

(5.)	Hold	2 minutes at 50°C
(6.)	Hold	30 minutes at 60°C
(7.)	8 cycles	10 seconds at 95°C, 10 seconds at 52°C, 10 seconds at 72°C
(8.)	23 cycles	10 seconds at 90°C, 10 seconds at 55°C, 10 seconds at 72°C
(9.)	Hold	15 minutes at 72°C

(10.) In the CYCLE programs the ramp time and allowed setpoint error should be left at the default settings of 0:00(which is the maximum rate) and 2'C.respectively. Link the 5 programs together into a METHOD program.

(11.) Start the method program. The program runs for approximately one hour and 30 minutes.

(12.) Remove the reaction tray form the thermal cycler beyond the end of the final Hold period. Do not allow the tubes to remain in the thermal cycler beyond the end of the final Hold period and do not extend the final HOLD program beyond 15 minutes. **Do not bring amplified DNA into the other areas. The amplified material should be considered to be significant potential source of DNA contamination.**

(13.) Remove the caps from the reaction tubes carefully so as to avoid aerosols of the amplification products. Immediately pipette 100µl of MONITOR Denaturation Solution into each reaction tube using a multichannel Pipettor, and mix carefully pipetting up and down at least 5 times. Preferably use the AMPLICOR Electronic IMPACT Pipettor set on Program 1 (see separate procedure).

(14.) The detection amplicon can be held at room temperature no more than 2 hours before proceeding to the detection reaction. If the detection reaction can not be performed within this time, re-cap the tubes and store the denatured amplicons at 2 to 8°C for up to one week.

Detection

(1.) Warm all reagents and amplicons to room temperature prior to proceeding with the detection.

(2.) Prepare a sufficient amount of working Wash Solution (dilute 1 part of the 10X with 9 parts of distilled or deionized water). This working Wash Solution is stable for up to 2 weeks at room temperature.
(3.) Allow the microwell detection plate to warm to room temperature before removing it form its foil pouch.
(4.) Add 100µl of MONITOR Hybridization Buffer to each well using Program 2 on the IMPACT Pipettor.
(5.) Add 25µl of the denatured amplicons to the wells of row A of the detection plate, mix up and down 10 times (twice). Use aerosol resistant plugged pipette tips. Make serial 5-fold dilutions in wells B through F as follows: transfer 25µl from row A to B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25µl. Discard pipette tips. This may be done using the IMPACT Pipettor Program 3 two times, where 25µl is transferred and mixes by pipetting 60µl up and down 10 times, and aspirates 25µl.
(6.) Add 25µl of the denatured amplicons to the wells of row G of the detection plate in the same manner as described in step 5. Mix as described in step 5 and transfer 25µl from row G to row H, again, mix as described in step5; remove and discard 25µl from row H along with the pipette tips.
(7.) Cover the plate and incubate it for 1 hour at 37°C (± 2°C).
(8.) Wash the entire detection plate 5 times with the working Wash Solution and an automated microplate washer. The microplate washer should entirely fill each well (400-450µl), allow each row (or column) soak for 30 seconds, then aspirate the entire contents of each well before proceeding to the next cycle.
(9.) Add 100µl of Avidin-HRP conjugate to each well, cover plate and incubate for 15 minutes at 37°C (±2°C).
(10.) Wash the plate as described in step 8.
(11.) Prepare the working Substrate solution by mixing 4 parts of substrate A with 1 part of substrate B. Protect the working solution from direct light.
(12.) Pipette 100µl of the working Substrate solution to each well and allow the color (light blue to dark blue) to develop for 10 minutes at room temperature in the dark.
(13.) Add 100µl of Stop Reagent to each well (blue color will turn to a yellow color).
(14.) Measure the optical density at 450 angstroms within 10 minutes of adding the Stop Reagent otherwise less dilute wells will precipitate resulting in a lower OD.
(15.) Decontaminate work area with a 1:10 bleach solution.

CALCULATIONS:

MANUAL CALCULATIONS

(1.) For each specimen, control or standard, choose the appropriate HIV well, as follows:
  a. The HIV wells in rows A through F represent neat and 5-, 25-, 125-, 625-, and 3125-fold serial dilutions of the amplicons, respectively. The absorbance values should decrease with the serial dilutions, with the highest value for each test in row A and the lowest value in row F.
  b. Choose the well where the raw OD is in the range of 0.200 to 2.0 OD units. If more than 1 well is in this range, choose the well with the larger dilution factor (i.e., the smaller OD).
  c. If any of the following conditions exist see Unexpected Results, below: all HIV OD values <0.200; all HIV OD values >2.0; HIV OD values are not in sequence (i.e., the OD values do not increase from well A to well F).
(2.) Subtract a background value of 0.070 OD units from each of the selected HIV OD values.
(3.) Calculate the "total HIV OD" by multiplying the value derived in step 2 by the dilution factor associated with that well.
(4.) For each specimen, control or standard, choose the appropriate QS well, as follows:
  a. The QS wells in rows G and H represent neat and 5-fold dilutions of the amplification products, respectively. The absorbance value in row G should be greater than the value in row H.

b. Choose the well where the OD is in the range of 0.300 to 2.0 OD units. If both wells are in this range, choose well H.

c. If one of the following conditions exist, see Unexpected Results, below: both QS OD values <0.300; both QS OD values >2.0: QS OD values are not in sequence (i.e., the OD values do not decrease from well G to well H).

(5.) Subtract a background value of 0.070 OD units from each of the selected QS OD values.

(6.) Calculate the "total QS OD" by multiplying the value derived in step 5 by the dilution factor associated with that well.

(7.) Calculate HIV-1 RNA Copies/mL plasma as follows:

HIV-1 RNA Copies/mL Plasma = ("total HIV OD"/"total QS OD") X Input QS Copies per reaction X 40

Computerized (Manual – LDMS) Calculations

(1.) On LDMS System, go to the assay module.

(2.) Click on the (+) sign next to Viral Load RNA from the Assay lists box.

(3.) Then click on the New Run/Not Setup button on upper right side of Search Criteria box.

(4.) Click on the Select button at the bottom of the Assay Selection screen. The Filters/Criteria screen appears.

(5.) At this time it is unclear whether the Filers/Criteria screen will be used. This section is used to pull pending viral loads from the database by using specific parameters. . At this point we will not be using the button "All Pending Specimens" because the list pulled would be too numerous to look through. The best suggestion at present to find specimens is to select the group from the Group combo box. Then narrow your search, create query statements using the Field, Operator and Value combo boxes.

(6.) Click the Find Specimens button at the bottom of the screen. This will take you to the Specimens Found screen. Use the shift or ctrl keys to select each specimen you want to place in the assay or use the buttons at the bottom of the Specimens Found screen. After selecting specimens, click the Add to Plate button at the bottom of the screen. LDMS will automatically move to the Plate Preview screen and load the chosen specimens onto the plate in the order determined by the user.

(7.) Repeat the process from step five if more than one group needs to be put on the run.

(8.) In the Plate Preview screen the user can add and delete plates to run; move, delete add and modify specimens; move, add, and modify controls; save the template to run later; click the Run Now button to run the assay.

(9.) After saving the assay od's from the LDMS Remote Reader Software, it can be read into the LDMS database and merged with the template previously set up. Put the disk into the A: drive. Go to the Assay module again.

(10.) Click the Viral Load RNA.

(11.) Click on the Runs Not Performed button.

(12.) Enter the run id of your assay in the Run Id field.

(13.) Click on your assay to select it, then click on the Select button at the bottom right of screen. The Preview tab will activate.

(14.) Click on the Preview tab, then click the Run Now button the bottom of the screen.

(15.) The input Copies dialog box will appear. Enter a value in the edit field, then click on the ok button on the dialog box.

(16.) A file dialog box will appear. Open the A:/devdata folder, then click on your file.

(17.) Click on the Open button on the File dialog box, and LDMS will begin reading the raw data from the disk in your A: drive.

(18.) When the LDMS is finished reading the assay data from your remote reader disk, the Results screen will appear in Plate Results view displaying the raw data from your remote assay run. Click on the Calculated Results button to view results on a specimen by specimen basis.

RESULT CRITERIA

(1.) If all of the HIV wells have OD values less than 0.200, but the QS wells have the expected values, use 0.200 as the HIV OD, calculate the result, and report the result as "Not detected, less than" the calculated value.

(2.) If all the HIV wells have OD values greater than 2.0, but the QS wells have the expected values, either an error occurred in the test, or the HIV copy number is above the dynamic range of the assay. Report the result as "Not determined". Repeat the entire assay, making a 1:50 dilution with HIV negative human plasma. Calculate the results as above and multiply by 50.

(3.) If the HIV wells do not follow the pattern of decreasing OD values from well A to well F, and error in dilution may have occurred. Examine the data according to the following criteria to determine if an error occurred. If an error occurred, report the result the as "Not determined" and repeat the entire assay including specimen preparation; otherwise, calculate and report the result as described above:

a. The OD values for HIV wells should follow a pattern of decreasing OD values with increasing Dilution Factor 9 (i.e., from well A to F), expect for well that are saturated and wells with background OD values.

b. In reactions containing high HIV-1 RNA copies per ml, wells A, B, and C can become saturated turning a greenish – brown color prior to the addition of Stop Solution and a brown color after addition of Stop Solution, resulting in lower OD. These results are valid even though the HIV wells do not have decreasing OD values from wells A through F.

c. In reactions containing low HIV-1 RNA copies per ml, wells B through F may contain background OD values. Such tests are valid even thought he HIV wells do not have decreasing OD values from well A through F. Wells with OD values (>2.3) may be saturated and wells with very low OD values (<0.1) are close to background. These wells may not follow a pattern of decreasing OD values from well A to well F.

d. All well with OD values </=2.3 and >/=0.1 should follow a pattern of decreasing OD values from well A to well F. If OD values do not follow a pattern of decreasing OD values from A to F then an error occurred.

(4.) If both QS wells have OD values less than 0.300, either the processed sample was inhibitory to the amplification, or the RNA was no recovered from the sample. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.

(5.) If both QS wells have OD values greater 2.0, an error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.

(6.) If the absorbance well H is greater than the absorbance of well G, and error occurred. The result for that specimen is invalid. Repeat the entire test procedure including specimen processing.

(7.) The assay is used for research purposes and for use in clinically monitoring viral loads.

(8.) Examples of unexpected results below:

ROW	Dilution Factor	Example 1	Example 2	Example 3
A	1	2.610	2.564	0.812
B	5	2.461	2.684	0.0161
C	25	3.112	2.432	0.055
D	125	2.668	1.032	0.064
E	625	2.984	0.287	0.079
F	3125	1.568	0.074	0.052

Interpretation:	Very high titer specimen. Not an error.	High titer specimen. Not an error.	High titer specimen. Not an error.	Low titer specimen. Not an error.

REPORTING RESULTS:

Results will be reported on the data fax form.

PROCEDURE NOTES:

(1.) Heparin should not be used as an anticoagulant as it inhibits PCR.
(2.) Residual ethanol left on the pellet will inhibit the amplification.
(3.) Due to the high analytical sensitivity of this test and the potential for contamination, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures.
(4.) All reagents should be closely monitored for purity. Discard any reagents that may be suspect.
(5.) Workflow in the laboratory should proceed in a uni-directional manner, beginning in the reagent preparation area, then the specimen preparation area, onto the Amplification/detection area.
(6.) Supplies should be detected to each activity and must not be used for other activities or moved between areas. Equipment and supplies used for reagent preparation/specimen preparation activities must not be used for pipetting or processing amplified DNA or other sources of target DNA.
(7.) Gloves must be worn in each and changed before leaving that area.
(8.) Good laboratory technique is essential to the proper performance of the assay.

LIMITATIONS OF PROCEDURE:

(1.) The presence of AmpErase in the AMPLICOR HIV-1 MONITOR Master Mix reduces the risk of amplicon contamination. However, contamination from HIV positive controls and HIV positive clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified above.
(2.) Use of this product should be limited to personnel trained in the techniques of PCR.
(3.) Only the Perkin-Elmer GeneAmp PCR System 9600 or GeneAmp 2400 thermal cyclers can be used with this product.
(4.) Monitoring the effects of antiretroviral therapy by serial measurements of plasma HIV-1 RNA has only been validated for patients with baselines viral loads >= 25,000 copies/ml.

The performance of the Amplicor HIV-1 Monitor Test has only been validated with HIV subtype B specimens.

Appendix C: PBMC Isolation and Cryopreservation /ELISPOT Assay Protocol

General Lab Supplies and Reagents Suggested for Lymphocyte Isolation and Cryopreservation:

Supply	Description	Suggested Vendor/Catalog #
50 ml tubes:	Polypropylene disposable tube (**DO NOT USE POLYSTYRENE)**	Fisher Scientific/05-539-6
15 ml tubes	Polypropylene disposable tube (**DO NOT USE POLYSTYRENE**)	Fisher Scientific 05-5395-5
Pipettors	20, 200, 1000 µL pipettors recommended	Ranin/P-20, P-200, P-100
Pipette Tips	250 µL capacity in racks of 100/rack	Ranin/ RT-20 *
Pipette Aid	NOTE: Available in 110V, 220V	Drummond/VWR/4-000-111(110V), 4-000-220 (220V)
Pipettes	2 ml plastic individually wrapped-sterile 10 ml plastic individually wrapped-sterile 25 ml plastic individually wrapped-sterile	Fisher/29442-418 Fisher/29442-430 Fisher/29442-436
Hemocytometer	Hausser Scientific for Phase Microscope	VWR/15170-079
Hemocytometer Cover Slips	Octagonal cover slip	VWR/151-70-296
Trypan Blue	For assessment of cell viability	Life Technologies/152250061
Ficoll-Paque™	Store in dark bottle at room temperature	Amersham-Pharmacia/17-1440-03
RPMI 1640 1X with Glutamine	Liquid Media 10 x 500 mls	Life Technologies/11875119
Penicillin /Streptomycin	Store in frozen aliquots of 5-10mls at –20°C	Life Technologies catalog/15140-122. Stock solution is 100X.
Fetal Bovine Serum	Store at –70°C	Price compare
DMSO	Six month shelf life	J.T. Baker/9224-01
10X PBS	Dilute to 1X with ddH_20	Life Technologies catalog # 14190-144
Nalgene “Mr. Frosty”	Used for control rate freezing. Store at 4°C when not in use.	Curtis Matheson Scientific/288-383
Cryogenic Freezing Vials	2 mL polyethylene vials with screw cap	Fisher Scientific/12-565-171N

Purple Top EDTA Tubes	10ml purple top EDTA vacutainer tubes	VWR/VT6457
Green Top Tubes	10 ml Sodium Heparin vacutainer tubes	VWR/VT6480
96-well plate	Round Bottom styrene plates	VWR 29442-398

REAGENTS TO MAKE

Solution Name:	**Recipe:**	**How to Make:**
FCS		Heat FCS at 56°C for 30 minutes to inactivate complement proteins
Culture Media (R-10)	RPMI/ 10% FBS/1X Pen/Strep.	To 500 ml bottle of RPMI 1640 1X Media, add 56 mls of FCS and 5.6 mls of 1X Pen/Strep. Store at 4°C in dark.
Culture Media (R-1)	RPMI/ 1% FBS/1X Pen/Strep.	To 500 ml bottle of RPMI 1640 1X Media, add 5 mls of FCS and 5mls of 1X Pen/Strep. Store at 4°C in dark.
Freezing Media (FM):	90% heat-inactivated FCS/10% DMSO.	Keep at 4°C in dark bottle for up to one month.
Washing Media (WM)	PBS/1%FCS/1%Pen-Strep	To 500ml bottle of PBS add 5 ml of FCS and 5 ml of Pen-Strep.

LYMPHOCYTE ISOLATION AND CRYOPRESERVATION METHOD:

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NOTES:
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- Do not refrigerate or freeze vacutainer tubes. If they arrive cold (from weather-related transport), allow them to come to room temperature.
- Store tubes at room temperature prior to processing.
- Make sure tubes are adequately mixed by inverting the tubes gently several times prior to Ficoll procedure.
- Both Ficoll and DMSO are toxic to cells. It is necessary to work quickly and wash well to minimize cell loss.

Supplies needed:
50ml sterile conical polypropylene tubes.
Sterile 25 and 10ml pipettes
Ficoll Hypaque (1.077-1.080 g/ml)
Wash media
Freezing media
Sterile Cryovials

96-well counting plate/small tubes for counting cells
Mr. Frosty
Hemocytometer
P20 pipettor, tips

1) 50 ml tubes containing ficoll can be prepared in advanced and store at room temperature. The blood:ficoll ratio should prepared according to the manufacturer's instructions. Blood can be diluted 1-1.5 with WM to bring the volume up when necessary.

2) Remove blood from vacutainer tube using a 10ml pipet, and carefully over-layer the blood onto the Ficoll. Do not disturb or mix tube once Ficoll has been added. This will destroy the gradient layer.

3) Centrifuge for 25 minutes at 400 x g at room temperature.

4) Carefully pipette buffy coat layer with a 10 ml pipette being careful not to take any of the RBC pellet (should pull up about 7-8 ml). Pipette cells into a fresh 50 ml tube, and bring up to 45ml with WM.

5) Centrifuge for 15 min at 400 x g.

6) Discard supernatant. Resuspend cell pellet gently by adding 5 ml of WM and mixing well with same pipette. Add additional 35 ml of WM, cap tube and invert several times.

7) From the well-mixed cell suspension take out 20µl using P-20 pipettor and put in 96-well counting plate. Re-cap tube and centrifuge for 10 min at 400 x g.

8) While cells are spinning, count cells:

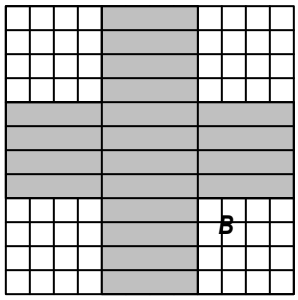
Cell counting:

The following steps are meant to be used as a guideline for lymphocyte counting using a hemocytometer. Counting lymphocytes using a hemocytometer is recommended over the Coulter Counter method so that cell viability and morphology can be viewed by eye.

- After resuspending cells, take a small amount (20µl) of the cell suspension and transfer to a well in the 96 well plate or a small tube.
- Add an equal volume of Trypan Blue dye to dilute the cells 1:2. Mix the cell suspension well and add to the hemocytometer by placing the tip of pipette to the space between the groove in the hemocytometer and the cover slip. Push the pipette plunger just enough to let the cell suspension be taken up by capillary action until the counting grid is just covered. DO NOT add too much liquid.
- When using Trypan Blue exclusion dye, live cells are white and dead cells are blue. **Note:** it is sometimes difficult to distinguish red blood cells (RBCs) from lymphocytes.
- Using the picture below, count cells that are found in squares A and B. You should count cells within the triple line boundary; cells can be touching this boundary, but should not cross the boundary.
- After the both A and B squares have been counted, add the two together and multiply that by 10^4. This calculation will give you the number of cells per ml. To obtain total cell number, multiply the number of cells/ml x total volume of cell suspension.

- **Example:** if you count 34 cells in A and 25 cells in B, them $34+25=59x10^4=5.9x10^5$ cells/ml of sample. If the sample is in 10 mls of media, then the total number of cells is $5.9x10^6$.

- Clean the hemocytometer and cover slip with bleach and 70% ethanol.



9) Resuspend pellet to a final cell concentration of 10^7 cells/vial in FM.

10) WORK QUICKLY AT THIS POINT TO GET CELLS IN FREEZER!!

11) Aliquot 0.5 to 1.0 mL of the cell suspension per cryovial. Be sure cryovial caps are securely tightened so that liquid nitrogen does not leak in. This can cause the vial to burst upon thawing.

12) **Immediately** place cryovials in isopropanol freezing container (i.e. "Mr. Frosty") and transfer to a -70°C freezer. Alternatively, place cryovials in a Cryomed controlled rate freezing chamber, lowering the temperature at -1°C per minute to -70°C.

13) Transfer cryovials to liquid nitrogen after 24 hours at -70°C.

Quality Control for Lymphocyte Isolation

- During the lymphocyte isolation procedure, the cells must go through a series of washes to remove residual Ficoll, which is toxic to cells.

- The QC for this step will be recorded on the specimen collection form as the lymphocyte viability (# live cells / # live cells + # dead cells).

- This will determine the quality of blood layering onto Ficoll, quality of isolation and collection of a buffy coat layer, and quality of washing Ficoll from cells. All of these conditions are necessary to prepare cells for optimal cryopreservation.

THAWING PROCEDURE:

1. Transfer cryovial from liquid nitrogen to a 37°C water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.

WARNING: Some cryovials have been reported to explode during the thawing process. To minimize this risk, use only unbreakable polyethylene vials for storage in liquid nitrogen (see above under materials/reagents). If polypropylene tubes are used, they should be sealed in cryoflex tubing.

2. Hold the cryovial in the surface of the water bath with an occasional gentle "flick" during thawing. Do not leave cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly). When a small bit of ice remains in the cryovial, transfer the cryovial to the biosafety hood. Dry off the outside of the cryovials before opening to prevent contamination.

3. Add R1 drop wise into the cryovial containing the cell suspension up to a volume which doubles the original volume (e.g., add 1 mL of media to a vial containing 1 mL cell suspension).

4. Transfer the cell suspension to a 15 ml conical bottom centrifuge tube containing 8 mL of warmed R1; wash twice by centrifugation, gently resuspending the cells between washes.

5. Determine cell number, record both alive and dead, to determine cell viability. Resuspend cells at desired concentration for assay to be used.

ELISPOT ASSAY for detection of secreted IFN-g

Products needed for the assay:

Products	**Company**	**Catalog #**
96 well/plate MILLIPORE Multiscreen Plates	MILLIPORE	MAHAS4510
Primary AB: Anti-IFN? Mab 1D1K	MABTECH	3420-3
Secondary AB: Anti-IFN? Mab 7B61 Biotinylated	MABTECH	3420-6
Vectastain	Vector Laboratories	PK6100
Stable DAB	Research Genetics	750118
PBS w/o Ca and Mg	GIBCO	14190-136
Tween- 20	Biorad	170-6531
RPMI 1640	Life Technologies	11875119
DMSO	GIBCO	61870-036
PHA-P 5mg/vial	SIGMA	D-2650
10X PBS	Gibco BRL	14200-075
Plate sealer (to hold plate membranes)	Millipore	MATA HCL 00
Filters (to punch out membranes)		
Media		
RPMI 1640 supplemented with 10% FCS (R10)		
RPMI 1640 supplemented with 20% FCS (R20)		
1X PBS-Tween supplemented with 0.1% Tween 20 (PBS-T)		
DMSO		

Procedure

Day 1:

1. Dilute primary ab for plating: The stock solution of the primary anti-IFN? monoclonal antibody is 1mg/ml. The working concentration is 10ug/ml. Add 10ul of the stock to every 1 ml of 1X PBS (eg 50 ul anti-IFN? and 5 mls of PBS to coat one plate). Pipet 50ul into each well of HA plates.
2. Store at 4°C overnight wrapped to prevent evaporation.
3. Thaw PBMC to be tested and record viability and recovery. Resuspend at 2-4x10^6 cells per ml of R-20 in a 50ml conical tube with the cap loosened. Do not exceed 5mls/50 ml tube. Let the cells rest overnight in CO_2 incubator.

Day 2

1. Wash antibody coated plates 4x with 1X PBS.
2. Block plates with R10 for at least 30 minutes at 37°C or until the cells are ready to be plated.
3. Prepare the cell dilutions: Count cells (record live and dead cells), spin cell suspension at 400 x g and resuspend the cells at 1x10^6 cells/ml. This will give 1x10^5 cells/100ul.
4. Prepare the peptide pools/matrix dilutions: TBA
5. ***Discard the blocking medium from the plates.***
6. Pipet 100ul of the 1x10^6 cells/ml to each well. Final concentration of cells is 10^5 per well.
7. Add 100ul of R-10/DMSO to each of the mock wells.
8. Add 100ul of the R-10/PHA/DMSO dilution to each of the PHA wells.
9. Add 100 ul of the peptide dilutions to the peptide wells.
10. Incubate for a minimum of 12h or up to 18h at 37°C in 5%CO_2.

Day 3

1. Discard the cell suspension
2. Wash by pipetting 100ul of PBS-T (PBS containing 0.1% Tween 20 – Sigma) into each well. Discard the PBS-T by flicking plate into large container in hood. Blot plate on paper towel. Repeat 4x.

3. Add 50µl/well of diluted secondary antibody (dilute stock antibody 1:1000 in 1X PBS to 1µg/ml) Incubate at room temperature for 2h.
4. Thirty minutes before the incubation has ended, make the Avidin bound Biotinylated HRP (Vectastain Elite Kit) by adding 2 drops of reagent A + 2drops of reagent B to 5 ml PBST and allow to stand at RT for 30 min to let complex form).
5. Wash the plate 4x in PBS-T.
6. Add 50µl to each well of Avidin bound Biotinylated HRP and incubate for 1h at room temperature.
7. Pipet out an appropriate amount of stable DAB into a conical tube and let stand at room temperature (100µl/ well; 10mls/plate).
8. Wash the plates 4x in PBS-T. Add 100µl / well of stable DAB /well and incubate for no more than 2 minutes.
9. At 2 minutes, discard the Stable DAB from the wells and quickly 1dd 100µl/well of tap to stop the reaction. Discard. Rinse plate under running tap water several times to prevent over-development.
10. Count the spots with a stereomicroscope (Stemi 2000 stereo microscope, Carl Zeiss, Inc. New York) under magnifications of 20-40x.
11. Only spots with a fuzzy border and a brown color are to be counted. (A positive test is 5 spot forming colonies per 10^5 cells after deducting the control).
12. After counting, peel off the plastic backing on the plate and stick a sheet of Elisa plate sealer onto the membranes. Using a filter, punch out each well individually to insure that the membrane does not break when it is transferred to the sticky plate sealer. Either place the plate sealer cover back onto the plate sealer or store in a page protector until transport to the core laboratory for computer counting.