AIDS in India – A Regional Symposium-Workshop

on

Research, Trials and Treatment

July 31st to August 6th 2004

***Organized by*:**
AIDS International Training and Research Program (AITRP) of the Albert Einstein College of Medicine (AECOM), Bronx, New York, USA
Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore
National Institute for Mental Health and Neurosciences (NIMHANS), Bangalore

***With financial support from*:**
Center for AIDS Research, AECOM, Bronx, New York
National Institute of Mental Health, NIH, USA
National AIDS Control Organization, New Delhi, India
Indian Council of Medical Research, New Delhi India
Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore

Table of Contents

Program at a glance

31st July	-	Laboratory Workshop Day 1 *JNCASR* *Lab sessions: Drug susceptibility measurement, ELISPOT, Detection of M. tuberculosis via reporter phages, Reporter cell lines for HIV.*
1st August	-	Laboratory Workshop Day 2 *AM Session: NIMHANS* Lab sessions: Viral load and CD4 measurements *PM Session: JNCASR* *Lab sessions: ELISPOT assay, Use of lentiviral vectors for gene therapy*
2nd August	-	Symposium Inauguration - *JNCASR* *Plenary Session* *Symposium Session I – HIV/AIDS* *Symposium Session II – Prevention, Vaccines and*

Program in Full

31st July to 7th August 2004

7/31/04, Saturday

Venue: **Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR)**

Registration and Laboratory workshop

8:30AM	Registration
9:00AM	Welcome
9:30AM	Tea
10:00AM	Laboratory Session 1/Drug susceptibility in HIV (Instructor: Pheroze Joshi)
12:00 Noon	Laboratory Session 2/Elispot assay (Instructor: Amara Rama Rao)
1:00 PM	Lunch
2:00PM	Laboratory Session 1b/Drug susceptibility (…continued)
2:30PM	Laboratory Session 2b/Elispot assay (…continued)

8/02/04, Monday
Inauguration, Plenary and Symposium sessions I and II
Venue: **Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR)**

Inauguration

8:30AM — Welcoming Remarks
Udaykumar Ranga
Assistant Professor, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore

8:35AM — Introductory Remarks
Vinayaka R. Prasad
Program Director, AIDS International Training and Research Program Albert Einstein College of Medicine, Bronx, NY, USA

8:40AM — Inaugural Address
Dr. C. N. R. Rao
Professor, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore

9:10AM — Plenary Lecture
Mark A. Wainberg
McGill University AIDS Centre, Montreal, Canada
"The Critical Need for HIV/AIDS Prevention Research in Developing Countries"

11:05AM	Shahid Jameel *International Center for Genetic Engineering and Biotechnology, New Delhi* "Role of HIV-1 Nef protein in viral pathogenesis"
11:25AM	Sundarasamy Mahalingam *Centre for DNA Fingerprinting and Diagnostics, Hyderabad* "Lentiviral accessory proteins: Lead roles for the supporting cast"
11:45PM	Debashis Mitra *National Centre for Cell Science, Pune* "Mitochondrial dysfunction in HIV induced T cell apoptosis"
12:05PM	Gopalan Sridharan *Christian Medical College, Vellore* "Certain aspects of the immunological profile of South Indian HIV infected individuals"
12:25PM	Robin Mukhopadhyaya *Cancer Research Institute, Mumbai* "A first-generation lentiviral vector system for gene delivery, derived from an Indian HIV-2 isolate: In vitro studies"
12:45PM	Ernest Drucker *Albert Einstein College of Medicine, Bronx, New York* "The Role of Unsterile Medical Procedures in the Origin of the AIDS Epidemic"

3:15PM	Vijay Mehra *International AIDS Vaccine Initiative, New York, NY, USA* "IAVI efforts in accelerating AIDS vaccine development"
3:35PM	Coffee Break
3:50PM	Ramesh Paranjpe *National AIDS Research Institute, Pune* "Cytolytic T Lymphocyte response in Subtype C infected Indian patients"
4:10PM	Shekhar Chakraborty *National Institute of Cholera and Enteric Diseases, Calcutta* "Characterization of HIV strains circulating in Eastern and Northeastern India: Development of a Candidate Vaccine"
4:30PM	Ramarao Amara *Emory University, Atlanta, GA* "DNA/MVA Vaccines for HIV/AIDS"
4:50PM	N. K. Mehra *All India Institute of Medical Sciences, New Delhi* *"Immunomodulatory genes and HIV Infection"*
5:10PM	Laurie Baumann *Albert Einstein College of Medicine, Bronx, NY*

8:50AM	Plenary Lecture William Jacobs, Jr. *Howard Hughes Medical Research Institute, Albert Einstein College of Medicine, Bronx, NY, USA* "The Primary Mechanism of Attenuation of BCG: Can we do better?"
9:20AM	S. Vijaya *Indian Institute of Science, Bangalore* "Can the human immune response guide the design of better TB vaccines?"
9:40AM	Jaya S. Tyagi *All India Institute of Medical Sciences, New Delhi* "Two-component signal transduction systems of *M. tuberculosis*"
10:00AM	Anil Tyagi *University of Delhi, South Campus, New Delhi* "*Mycobacterium tuberculosis* - Gene regulation, Pathogenesis and TB vaccine"
10:20AM	Coffee Break
10:50AM	Manikuntala Kundu *Bose Institute, Calcutta, India* "*Mycobacterium avium*-induced signaling in host macrophages"
11:10AM	Soumya Swaminathan *Tuberculosis Research Centre, Chennai*

1:45PM	Richard Price *University of California, San Francisco, CA* "AACTG5199: A Protocol for an International Study of the Impact of Antiretroviral therapy on Neurological Function in Resource Restricted Settings"
2:05PM	Bruce Brew *St. Vincents Hospital, Sydney, Australia* "The Asia Pacific NeuroAIDS Consortium: Strengths and weaknesses"
2:25PM	Mahendra Kumar *University of Miami School of Medicine, Miami, FL USA* "Neurocognition in HIV infection in South India: Preliminary Data"
2:45PM	Coffee Break
3:15PM	P. Satishchandra *National Institute of Mental Health and Neurosciences, Bangalore* "Opportunistic infections in the nervous system secondary to retroviral infections"
3:35PM	S. K. Shankar *National Institute of Mental Health and Neurosciences, Bangalore* "Spectrum of pathological lesions in HIV/AIDS in India"
3:55PM	Uday Kumar Ranga *Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore*

9:45AM - 10:00AM	*Tea Break*
10:00AM – 11:00AM	Antiretroviral Treatment Modalities - 1 M. Markowitz, Aaron Diamond AIDS Research Center, NY, USA K. Satish, Wokhart Hospital, Bangalore
11:15AM – 12:15PM	Fundamentals of Epidemiology and Statistics -1 Paul Marantz, Albert Einstein College of Medicine, Bronx, NY, USA
12:30PM – 1:30PM	Lunch
1:30PM – 2:30PM	Biology of HIV/AIDS Prevention Kenneth Mayer, Brown University, Rhode Island
2:45PM – 3:15PM	Coffee Break
3:15PM – 4:15PM	Behavioral Methods of AIDS Prevention- I Larie Bauman, Albert Einstein College of Medicine, Bronx, NY, USA Sunil Mehra, MAMTA, New Delhi
4:30PM – 5:30PM	HIV and Infectious Risks of Blood Transfusion-I Robert Reiss, New York Blood Center, New York, USA Latha Jagannathan, Bangalore Medical Services Trust, Bangalore Vasanthapuram Ravi, NIMHANS, Bangalore Nilkantrai Vaishnav, Bhavnagar Blood Bank, Bhavnagar, Gujarath

1:30PM – 2:30PM	Behavioral Methods of AIDS Prevention- 2 Larie Bauman, Albert Einstein College of Medicine, Bronx, NY, USA Sunil Mehra, MAMTA, New Delhi
2:45PM – 3:15PM	Tea Break
3:15PM – 4:15PM	HIV and Infectious Risks of Blood Transfusion - 2 Robert Reiss, New York Blood Center, New York, USA Latha Jagannathan, Bangalore Medical Services Trust, Bangalore Vasanthapuram Ravi, NIMHANS, Bangalore Nilkantrai Vaishnav, Bhavnagar Blood Bank, Bhavnagar, Gujarath
4:30PM – 5:30PM	Grant-Writing Workshop - 2 Madelon Halula, Mary Kirker, Priti Mehrotra & Paula Strickland, National Institute of Allergy and Infectious Diseases, NIH, USA
6:30PM	Dinner
7:30PM	Laboratory Session 7b/ Heteroduplex mobility shift assays Udaykumar Ranga, JNCASR, Bangalore

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8/06/04, Friday
Laboratory Workshop
Venue: **Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR)**

Workshop Materials

(For Round Table and the Laboratory Components)

B. *Round-table discussion series (August 3rd to August 5th)*

These are 2- session series covering basic-to-advanced discussion on core topics of interest to both clinicians and basic researchers. Each session is 60 minutes long, and begins with an introductory lecture followed by discussion by the participants.

Roundtable Assignments (Initial)

The assignments do not suggest that trainees only attend the assigned roundtables. All participants take part in all roundtables – but the assigned trainees are expected to take an in-depth interest in those roundtables. Please note that these assignments were made based on the organizers' perception of your expertise and/or based on your own information (in cases where the questionnaire was returned in a timely fashion). These assignments are not final. Participants are welcome to change them.

Ethics Macklin/Jesani

No one indicated ethics as their specialty. So, we have assigned all those whose interest was Vaccine trials (as this is the topic for the first of the two sessions) as well as some of the basic and clinicial researchers. However, we feel that the Ethics sessions are essential for all the participants.

Seetharamaiah Chittiprol
Suman Ranjan Das
Sushama Jadhav
Gurvinder Kaur
Sonali Kochhar
R. Sudheer Kumar
Swarali N. Kurle
Anita Mahadevan

M. R. Khan
Manish Kumar
Kaustuv Nayak
Yogesh Rajmane
Sharmila Reddy
R. Varadarajan

Prevention-Behavior Bauman/Mehra

Epidemiology Marantz

We noted that there were a handful of individuals specifically interested in epidemiology, but also a handful that were interested in population based research not titled epidemiology and also several who were interested in population-based behavioral studies. Since the underlying principle here was population-based research, I have grouped them both here. I will let the teachers and the participants sort out who wants to take a active role in which session.

Glory Alexander
Sanjay Desai
T. Padmaja
Hemraj Pal
D. M. Patel
S. Sakthivelponnusamy

Round Table Discussion Group 1

Grant-Writing Workshop

Session Leaders: Madelon Halula, Mary Kirker, Priti Mehrotra, Paula S.

Outline

The workshop will focus on funding opportunities for AIDS research, how to write a National Institutes of Health (NIH) grant, and management of a NIH grant.

Part I (Kirker, Halula, Mehrotra)
An introduction to NIH grant fundamentals and funding opportunities

Part II (Halula, Mehrotra, Strickland)

Round Table Discussion Group 2

Ethics in Clinical Trials and Scientific Research

Session Leaders: Dr. Ruth Macklin, Albert Einstein College of Medicine, NY
Dr. Amar Jesani, CEHAT, New Delhi, India

Outline

Session 1
Ethical principles in research involving human subjects
(a) Identification and description of ethical principles
(b) Application of principles in the design and review of research
(c) Challenges in applying ethical principles

Session 2
Ethics in research: special topics

Case Study 1

Testing a New Preventive HIV Vaccine

Viravax, a UK-based company has developed a vaccine against HIV that appears promising. Animal studies were very successful and phase I and phase II trials demonstrated that the vaccine was remarkably safe and that it produced significant antibody levels in essentially all of the volunteers. The company now wishes to begin phase III trials in a city in the Philippines where previous surveillance has identified a cohort of intravenous drug users (IDU) with a high rate of conversion to HIV-1. Such a study could be completed in two years. The Ministry of Health has expressed interest in having the study conducted and begins negotiations with Viravax. The vaccine, which is specifically directed against the strain that predominates in the Philippine IDU population, will be provided free by Viravax. Viravax will also cover the cost of conducting the study, which will be carried out by local and national researchers in the Philippines. In addition to the study costs, the company will provide all the laboratory equipment necessary to conduct the studies, ten computers for the institute in which the study is to be carried out, and two vehicles to visit the study sites. The company agrees that if the vaccine proves effective it will be given free of charge to the IDU population of the city and at cost to the country for five years.

Discussions are held regarding how to treat persons in either the vaccine or control group that are HIV+. This will be a randomized double blind prospective study with one group receiving the test vaccine and the other group receiving a placebo. All

conducted in the Philippines is because it is much less expensive to do, as triple therapy is not required (which would be necessary if the study was conducted in the UK). They feel that the study, as presently designed, would never be passed by either a UK government or university ethical review committee. Viravax counters that to use state-of-the-art therapy would in itself be unethical because it would be unsustainable in the Philippines and only one small group would have access to the best. In addition, the physicians in the Philippines would be unfamiliar with the therapy and unaware of all the possible side effects. And lastly, by offering the best care available in the world, the study would be giving an unfair inducement to participate. It would in essence be offering a form of coercion to receive the vaccine.

If, following the 3 years of the trial, this vaccine is found to be efficacious, the sponsor has agreed to enter into a discussion phase with the government and health authorities in the Philippines concerning provision of the vaccine to that country. It is recognized that a decision about how and for whom the vaccine should be provided will depend to large degree on results to be obtained from the study, such as side effects and degree of effectiveness. A promise about providing the vaccine cannot be made until this information is available. However, the sponsor is willing to ensure that the vaccine is made "reasonably available" at affordable rates to the individuals in that country for whom the vaccine is most appropriate.

Questions for Discussion:

1. Is the study unethical because participants are not being offered the best care available in the world if they should become HIV+?

screening process, before they are actually enrolled in the trial?. Should they be entitled to the same higher level of care and treatment that would be provided to people who seroconvert during the trial? Should they receive the standard treatment available to members of the population who were not screened for the trial? Or should they receive some intermediate level of care?

ACKNOWLEDGMENT: This case is adapted from an international research ethics course taught at the Harvard School of Public Health, Dr. Richard Cash, course director.

Case Study 2

Use of Placebo: TB Prevention in Uganda

From 1993 to 1995, researchers from Case Western Reserve University collaborated with researchers in Uganda to evaluate the safety and efficacy of three regimens to prevent tuberculosis in HIV infected adults. A randomized, placebo controlled clinical trial was conducted among 2,736 Ugandan adults who were HIV positive and either had a positive PPD skin test for tuberculosis or who were anergic based upon PPD and candida skin testing. Participants were randomly assigned to one of four regimens: placebo, isoniazid (INH) daily for six months, isoniazid, rifampin, and pyrazinamide daily for three months.

Case Study 3

The Role of Industry in Clinical Research

"Curae Mirae Pharma"* is a privately held biotechnology company which is registered to conduct business in a Latin American country. The company has never successfully developed a drug to the point of submitting a registrational dossier, (NDA), for approval and licensure anywhere in the world. CM Pharma was founded by a university researcher, Professor X, who is convinced that he has identified the cure for AIDS. Professor X was intent upon demonstrating the importance of his discovery to the world and prevailed upon the Minister of Science to ensure that a pilot study in HIV-infected subjects was fast tracked, receiving local approval to proceed without regulatory, local ethical review, or patient informed consent, "which would only have got in the way and slowed the project down." The "discovery" was based upon a high molecular weight, polar molecule which had to be administered parenterally. It had previously been used widely in the dyeing industry and was known to be a causative agent of acute myocarditis in both humans and various animal species. The drug has antiretroviral properties in 'in vitro' HIV-infected cell culture systems. The "discovery" was announced to the world and promptly disregarded by "outside" experts.

Professor X was convinced that, had his discovery come out of a US/European university institute, he would have received due credit. The board of CM Pharma

Now imagine that you are a senior clinical research scientist who has been employed as an expert consultant in pharmaceutical drug development to advise CM Pharma and implement the "proof of principle" phase I/II trial on behalf of the company.

Discussion Questions:
1. What ethical concerns might you have in this role and on behalf of your own employer, a Swiss consulting group of international repute?

2. What initial advice would you give CM Pharma and how would you advise your own management?

3. Should the trial take place? Why, or why not?

4. What could be done to ensure that the trial is conducted in either relevant local or international standards?

5. When difficulties are not easily resolved, what should your advice be to all parties?

*Curae Mirae Pharma is a fictitious company and the events depicted are unrelated to any company or individuals engaged in clinical research.

**This case study appears courtesy of Dr. Keith Bragman MD(Lond), FRCP, FRCPath, FFPM, Vice President, Head of Anti-Infectives Strategic Business Unit, Quintiles' Transnational Corporation, Europe. Dr. Bragman served as a member of

Round Table Discussion Group 3

Antiretroviral treatment modalities

Session Leaders: Marty Markowitz (Aaron Diamond AIDS Research Center, New York)

Outline:

Session 1
Introduction to Antiretroviral therapies
(a) Basics of HIV life cycle
(b) Classes of antiretrovirals and how they act
(c) Viral dynamics and the scientific rationale for combination therapy
(d) Scientific basis for drug resistance
(e) Testing for resistance

Session 2
Treatment guidelines

Round Table Discussion Group 4

Fundamentals of Epidemiology and Statistics

Session Leader: Paul Marantz (AECOM)

Outline

Session 1
Developing your research idea and designing your study
(a) What's the question?
(b) What sorts of study designs might you consider?
(c) Designing the study
(d) Calculating the sample size

Session 2

Round Table Discussion Group 5

AIDS Prevention -Biological

Session leaders: Kenneth Mayer (Brown University)

Outline of Session

The biology of HIV Transmission: Biological interventions that are available that might decrease transmission: STD control and antiretroviral therapy, including PEP; Biological interventions on the horizon: vaccines and microbicides

Round Table Discussion Group 6

AIDS Prevention -Behavioral

Session leaders: Laurie Bauman (AECOM)
Sunil Mehra (MAMTA)

Outline

AIDS Prevention - Behavioral
Theory and Research on HIV Prevention in Adolescence
How to Design Successful Programs to Prevent HIV Transmission in Adolescents

Round Table Discussion Group 7

HIV and Infectious Risks of Blood Transfusion

Session Leaders: Robert Reiss (NYBC), Latha Jagannathan (BMST),

Part 1.

Epidemiology of Transfusion Transmitted Viral Infections. (R. Reiss and N. Vaishnav)

- Epidemiological Studies of Transfusion Transmitted Infections (TTI) in USA.
- Models for Estimating Per Unit Infectious Risk of Donor Blood.
- Current Estimated Residual Incidence / Per Unit Risks in USAA.
- Epidemiological Issues in Transfusion Transmitted HIV and Other TTI in India.

Part 2

Modalities to Decrease Potential Infectivity of Allogeneic Donor Blood (L. Jagannathan and V. Ravi)

- Recruitment and Selection of Low Risk Donors.
- Pre-Donation Education and Screening

C. Laboratory Workshops (July 31st to August 6th)

Overview

Laboratory Session 1	**Drug susceptibility** Instructor: Dr. Pheroze Joshi
Laboratory Session 2	**Elispot assay** Instructor: Dr. Rama Rao Amara
Laboratory Session 3	**Detection of *M. tuberculosis*** Instructor: Dr. Vanaja Kumar
Laboratory Session 4	**Use of Reporter cell lines for HIV** Instructor: Dr. Udaykumar Ranga

Laboratory Session 1
Drug susceptibility
Instructor: Dr. Pheroze Joshi

Note: Printed version of the power point presentation of Dr. Joshi's talk is provided as part of a separate handout. The CD will include this presentation.

HIV DRUG SUSCEPTIBILITY ASSAY

I. PRINCIPLE

The in vitro drug susceptibility assay measures the extent that a drug inhibits HIV p24 antigen production by PBMC acutely infected with a viral isolate. It is performed in 96-well plates with a defined previously titrated inoculum of a clinical isolate to minimize inoculum effects. The infectivity of each clinical isolate is determined prior to drug susceptibility testing using a streamlined endpoint dilution assay that is analyzed by the Spearman-Karber statistical method.

Both the infectivity titration and susceptibility determination use PHA-stimulated PBMC from normal donors. After the infectivity of a virus stock is quantified, 1000 50% tissue culture infectious doses (TCID 50) per million PHA-stimulated PBMC is used as inoculum in a second set of in vitro infections. Infected wells (in the absence of drug and at each of a number of drug concentrations) are refed with a 50% medium exchange after 4 days of culture, and supernatant fluid is harvested after 7 days. HIV p24 antigen is quantified and the 50% inhibitory concentration (IC 50) of the drug is determined using the median effect equation.

II. SPECIMEN REQUIREMENTS

Cell-free supernatant is obtained from a positive HIV culture of patient PBMC, plasma, body fluid or tissue. This may originate from a qualitative macrococulture, a

only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened
bottles at room temperature; store aliquots at 4 C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20 C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37 C water bath, then heat-inactivate in a 56 C water bath for 30 minutes with occasional shaking. The level of H 2 O in the water bath should be as high as the level of the serum in the bottle. Store at 4 C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4 C and observe manufacturer's
outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price
for labs within the ACTG. Store at -20 C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:
To make 620 mL:
a. Add 120 mL heat-inactivated FBS to 500 mL of RPMI 1640 medium with

depending on drug (e.g., protease inhibitors should be stored at -85 C) in borosilicate glass vials. Avoid storing drug at low concentrations.
Drug-sensitive and drug-resistant isolates can be obtained from the NIH AIDS Research and Reference Reagent Program.

IV. SUPPLIES AND EQUIPMENT

Gloves
Disposable lab coat
Laminar flow hood (Class 2 biosafety hood)
Sterile 2, 5 and 10 mL pipette
Hemacytometer
96-well, flat bottomed tissue culture plates
Sterile 1.5 and 0.5 mL microcentrifuge tubes
20 µL, 200 µL, and 1000 µL micropipettor
Sterile 50 µL, 200 µL and 1000 µL pipette tip
Multichannel 50 µL, 200 µL micropipettors
Repeat pipettor and sterile tips
Borosilicate glass tubes
1% bleach or suitable disinfectant
Low speed centrifuge with O ring sealed safety cups
Compound microscope
CO 2 incubator (37 ±1 C with humidity)
37 C and 56 C water baths

PLATE FORMAT FOR HIV TITRATION

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
D	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
E	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

3. Add 150 µL Growth Medium to the wells labeled 4 -3 to 4 -8 (rows C to E, columns 4 to 9) with a multi-channel micropipettor.

4. Rapidly thaw an aliquot of the virus stock at 37 C in a water bath until only a small crystal of ice remains. Immediately dilute the sample 1:12 in Growth Medium (e.g., 0.1 mL of virus stock to 1.1 mL of culture medium) and transfer 200 µL to each well labeled 4 -2 (column 3 in rows C to E).

5. With a multi-channel pipette, transfer 50 µL from wells labeled 4 -2 to wells labeled

a. Scoring the HIV p24 antigen plate

	1	2	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	+	+	+	+	-	-	-	P	P	P
D	P	P	+	+	-	-	-	-	-	P	P	P
E	P	P	+	+	+	-	-	-	-	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

r = 0 0 1 2 3 3 3 = 12

b. Calculating TCID 50 /mL
xk = dose of highest dilution
r = number of "-" responses
d = spacing between dilutions
n = wells per dilution
r = sum of r

Spearman-Karber formula: $M = xk + d [0.5 - (1/n) (r)]$
$= 8 + 1 [0.5 - (1/3)(12)]$

3. With a multi-channel pipettor, add 100 µL Growth Medium to wells labeled "0" (C to E in column 3). Add 100 µL of 2X ZDV working solutions to their respective wells labeled with the 1X drug concentration (columns 4 to 8). The numbers in the illustration are the final concentrations of ZDV in µM. The remaining working solutions may be stored at -20 C until day 4 or made up fresh at the time. Peptides, such as protease inhibitors, should be prepared fresh each time. (See Plate Format below.)

PLATE FORMAT FOR DRUG SUSCEPTIBILITY TESTING

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	0	0.001	0.01	0.1	1.0	5.0	P	P	P	P
D	P	P	0	0.001	0.01	0.1	1.0	5.0	P	P	P	P
E	P	P	0	0.001	0.01	0.1	1.0	5.0	P	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

then thaw the 2X working solutions (or prepare fresh material). Add 0.5 mL of the 2X working solutions to 0.5 mL of Growth Medium to give 1X working solutions. Moving from left to right across the plate, mix, remove and discard 125 µL of cell suspension from the culture wells. Then add 150 µL of each of the 1X ZDV concentration (or other antiretroviral agent) working solutions to the appropriate wells (see plate format).

9. On day 6, prepare dilutions of the wells for HIV p24 antigen evaluation as follows:

a. With a multi-channel pipettor, add 205 TL of Growth Medium, followed by 25 µL of the manufacturer's disruption buffer containing Triton X-100 to the wells in columns 1 to 6 of rows C through H of a new 96-well, flat bottomed polystyrene plate. This will be the Dilution Plate and the 36 wells just pipetted will accommodate a 1:12.5 and 1:156 dilution of the Drug Susceptibility Plate.

b. Transfer 20 µL of supernatant from each of the drug susceptibility assay wells (rows C, D, and E of the Drug Susceptibility Plate) to their respective rows in the Dilution Plate (rows C, D, and E), using a multi-channel micropipettor (1:12.5 dilution). Mix and then transfer 20 µL from row C to row F, row D to row G, and row E to row H (total dilution of 1:156). Cover the plate with a 5 x 8 inch low-density polyethylene bag or similar product to prevent drying of the samples. Store the plate at -30 C or lower and run the HIV p24 antigen assay within 72 hours.

Systat (Systat, Inc., Evanston, IL) can solve for IC 50 and m simultaneously using nonlinear regression modeling. After the equation's constants (IC 50 and m) are determined, a curve of F a versus drug concentration can be constructed on a log-linear plot with drug concentration on the log scale of the x-axis.

2) logarithmic form of the equation can be used and a curve fit to the data points by linear regression:
Log (F a /F u) = m log [drug concentration] - m log [IC 50]
Fraction affected: (F a) = % reduction from untreated control x 0.01
Fraction unaffected: (F u) = % maximum x 0.01

b. "Dose effect analysis with microcomputers" by Chou and Chou (Biosoft, Ferguson, MO) calculates the IC 50 using linear regression to solve for the best fit of the data to this logarithmic form of the median effect equation. When actual values of F a /F u and drug concentration are plotted on log-log scales, the IC 50 is the drug concentration that corresponds to y=1. The Chou and Chou software plots log (F a /F u) versus log (drug concentration) on linear scales, and the IC 50 is the y intercept (since log 1 = 0). However, when using this method, F u cannot be zero and therefore must be changed to a consistent arbitrary number if no drug effect is seen at low drug concentrations.

c. Other software can be used: (a) for nonlinear regression modeling of the

that the day 7 harvest occurred after virus replication had peaked and that "breakthrough" of drug sensitive virus may have occurred in the presence of drug. For this reason, the protocol includes a microscopic examination of cultures for CPE on day 4.

B. The use of controls for donor PBMC variability in drug susceptibility testing is recommended.

1. A potential source of inconsistency in the drug susceptibility assay is the variability that is sometimes seen in the sensitivity of PBMC from different donors to support the growth of HIV. When feasible, it is advisable to use a designated pool of donors in whose cells HIV-1 is known to replicate well. The optimum is to use the same donor's cells for infectivity titration and susceptibility testing of a particular isolate. The following optional controls can help to assess the influence of donor PBMC variability on the replicative activity of HIV-1 and the eventual calculation of IC 50 .

a. With each batch of PBMC, inoculate 200 TCID 50 of a standard, well-characterized ZDV-resistant isolate (e.g., A018C) in triplicate wells containing 0, 0.1, and 1.0 μM ZDV. Expression of HIV-1 p24 antigen should be similar in all these wells for such a high-level ZDV-resistant isolate. Significant drug inhibition (>70% reduction at 1.0M relative to no drug, for example) raises the possibility of inadequate virus replication

HIV isolates arising after prolonged zidovudine therapy. J AIDS 5:749-59, 1992.

Richman DD, Johnson VA and Mayers DL. Measurement of susceptibility of HIV to antiviral drugs. Current Protocols in Immunology, Coligan JE, Kruisbeck AM, Margulies DH, Shevach EM and Strober W (eds)., Brooklyn, 1993.

Japour AJ, Mayers DL, Johnson VA, Kuritzkes DR, Beckett LA, Arduino JA, Lane J, Black RJ, Reichelderfer PS, D'Aquila RT, Crumpacker CS, the RV-43 Study Group and the AIDS Clinical Trails Group Virology Committee Resistance Working Group. A standardized peripheral mononuclear cell culture assay for the determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob Agents Chemother 37:1095-1101, 1993.

D'Aquila RT, Johnson VA, Welles SL, Japour AS, Kuritzkes DR, DeGruttola V, Reichelderfer PS, Coombs RW, Crumpacker CS, Kahn JO, and Richman DD for the AIDS Clinical Trials Group Protocol 116B/117 Team and the Virology Committee Resistance Working Group. Zidovudine resistance and human immunodeficiency virus type 1 disease progression during antiretroviral therapy. Ann Intern Med 122:401-8, 1995.

Laboratory Session 2
Elispot assay
Instructor: Dr. Rama Rao Amara

Summary: Enzyme Linked Immuno SPOT assay (ELISPOT) is used to *ex vivo* enumeration of antigen specific cytokine producing T cells. This assay is highly sensitive and quantitative. It is also easy to perform. Less expensive compared to the other quantitative assays such as Intracellular Cytokine Staining (ICS) assay and does not require costly equipment such as a flow cytometer.

PBMC PREPARATION

Reagents:

Cell Preparation Tubes (BD #362753)
Complete medium : RPMI-1640 with 10% FCS
ACK lysing buffer (Biosource International # P304-100)
Trypan blue solution (Cellgro # 25-900-CI)

Procedure:

1. Collect blood in BD VACUTAINER Cell Preparation Tubes (CPT) containing Sodium heparin.
2. Mix tubes well and centrifuge at 2000 rpm for 15-20 min at 25 ^{o}C. Carefully remove upper plasma layer without disturbing the PBMC layer.

ELISPOT PROTOCOL

Reagents:

Capture antibody (mouse anti-human α-IFN, Bio source International NON0096 or Pharmingen #554698)
Biotinylated anti-α-IFN (Diapharma group INC. M3420-6)
Avidin HRP (Vector Laboratories, A-2004)
Stable DAB (Research genetics 750118)
#Millipore Multiscreen 96 well filtration plate (Fisher NC9329127)
#Wash buffer : PBS-Tween (PBS with 0.05% Tween 20)
#Complete medium : RPMI-1640 with 10% FBS

Procedure:

Day 1:

1. Coat each well with capture antibody at a concentration of 5 μg/mL in PBS (50 μL per well) ONT at 4°C.

Day 2:

2. Wash plate two times with RPMI medium, add 200 μL of complete medium to

Laboratory Session 3
Detection of *M. tuberculosis using luciferase reporter phages*
Instructor: Dr. Vanaja Kumar

Protocol for Luciferase Reporter phage Assay

Materials:

Mycobacterial Strains:

1. *M.smegmatis* strain mc²155: A high frequency transformation derivative of *M.smegmatis* mc²6 (1)
2. *M.bovis* BCG sub-strains Pasteur, Copenhagen: attenuated vaccine strain of M.bovis BCG (Statens Serum Institute, Copenhagen, Denmark)

Growth of Mycobacteria:

1. Middlebrook 7H9 broth (10x stock): dissolve 47g Middlebrook 7H9 powder (Difco) in 920ml deionised water, add 40ml of 50% glycerol(2%v/v final concentration) and filter sterilize using 0.2µm filter membrane. Store in dark at 4°C. To prepare 7H9 broth, add 100ml of 10x 7H9 stock, 100ml of 10x ADS stock and 2.5ml of 20% Tween-80 (0.05% final concentration) to 800ml of deionised water. Filter sterilize through 0.2µm filter. Store in dark at 4°C.
2. Middlebrook 7H9 agar: add 15g agar Noble (Difco) to 800ml deionised water and autoclave. Cool to 56°C and add 100ml of sterile preheated 10X7H9 broth

Preparation of high titer phage lysates

1. Middlebrook 7H9 bottom agar: Add 18g Bacto-agar (Difco) to 900 ml of deionized water and autoclave. Cool to approximately 56^{o}C and then add 100ml of 10x7H9 broth preheated to 56^{o}C with stirring.

2. Top Agar: Add 3 g Bacto- agar (Difco), 1ml of 50% glycerol and 1 ml of 1 M $CaCl_2$ to 500 ml of deionozed water. Sterilize by autoclaving. Store batches of top agar in solidified form. Top agar can be stored in solidified form at 56oC for up to one week.

3.1M $CaCl_2$: Dissolve 55.5g $CaCl_2$ in 400ml deionised water. Adjust the volume to 500 ml with deionised water. Sterilize by autoclaving.

4. Phage buffer(MP buffer): Add 50ml of 1M Tris-HCL, pH 7.4, 10ml of 1M $MgSO_4$, 2ml of 1M CaCl2 and 30ml 5M NaCl to 800ml of deionised water. Adjust the volume to 1L with deionised water. Dispense into convenient volumes and sterilize by autoclaving.

5. Falcon tubes

6. 0.45µm filter units

Methods:

Preparation of high titer phage from plate lysate:

1. Plate the serial dilutions of phage with *M.smegmatis* mc^2155, incubate them as above and choose the ones giving confluent lysis on the bacterial lawn.
2. Cover the surface of each plate with 5ml MP buffer and for keep at 4^oC for about an hour.
3. Mix thoroughly and filter the lysate through a 0.45µm membrane and store at 4^oC.
4. Check for contamination by streaking a loop full of the lysate onto MH agar plate.
5. Determine the titer as described earlier.

Determination of the phage titer:

1. Prepare 10 fold serial dilution of the phage stock in 1ml of MP buffer generating serial dilutions of 10^{-7} to 10^{-1}.
2. Add 0.1ml of from each phage dilution to 0.3ml of the cells in a sterile tube.
3. Incubate the phage-cells mixture at 37^oC for 30 min to allow the adsorption of the phage to the bacterial cells.

Luciferin:

A working luciferin solution {0.33 mM D-Luciferin sodium salt; Analytical Luminescence Laboratory (ALL); Ann Arbour, MI} is freshly prepared from frozen (5 mg/ 1.5ml sterile DW) aliquots, ideally stored in 0.5ml quantities in 3 aliquots and diluted in 2ml of 500 mM sodium citrate buffer pH 4.5 made up to 20ml with sterile DW and stored at 4^{o}C protected from light. The Monolight 2010 luminometer (ALL) used 10s integration time, and produced background counts, with media alone, between 150 and 250 relative light units (RLU).

Luminometric detection by LRP

Luminometric drug susceptibility testing of M.tb cultures using luciferase reporter phage

The assay is performed as described by Riska and Jacobs (2, 3) with few modifications. A heavy suspension of each organism, equivalent to 4 McFarland units, corresponding to 1.2 X 10^{9} CFU/ml is prepared in 2 ml of 7H9 medium with glycerol and no Tween 80. (Alternatively, M.tb isolates grown in 7H9 broth with

Luciferase reporter phage assay DST

Control		**Test**
Plain 7H9 medium	350 µl	Drug containing 7H9 medium
M.tb growth/suspension	100 µl	M.tb growth/suspension
0.1M CaCl2	40 µl	0.1M CaCl2
phAE129 high titer lysate	50 µl	phAE129 high titer lysate

Incubate for 3 hours

100ul of phage-cell mixture + 100ul of luciferin working solution

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Measure RLU

Photographic detection by LRP

Drug susceptibility test by Bronx box

Mycobacterial cultures are washed twice with Middlebrook 7H9 broth without Tween80

References

1) Snapper,S.B., Melton,R.E., Mustafa.S., Kieser,T., and Jacobs, W.R., Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis . Mol.Microbiol.**4**, 1911- 1919.

2) Ramasamy, S., Musser, J.M. 1998**.** Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuberc.Lung.Dis. 79: 3-29.

3) Riska, P.F., W.R. Jacobs Jr. 1996. The use of Luciferase reporter phage for antibiotic susceptibility testing of mycobacteria. In Methods in Molecular Biology, Vol.101. Eds. T. Parish and NG Stokes. Humana Press, Inc., Totowa, N.J.. 431-455.

4) Riska, P.F., Y Su, S. Bardarov, L. Freundlich, G. Sarkis, G.F. Hatfull, G. Carriere, V. Kumar, J. Chan, W.R. Jacobs Jr. 1999. Rapid film-based determination of antibiotic susceptibilities of *Mycobacterium tuberculosis* by using a Luciferase reporter phage and the Bronx Box. J. Clin. Microbiol. 37**:** 114-1149.

Laboratory Session 4
Use of Reporter cell lines for HIV
Instructor: Dr. Udaykumar Ranga

Note: Printed version of the power point presentation of Dr. Ranga's talk is provided as part of a separate handout. The CD will include this presentation.

HIV-1 reporter cells

Indicator cell lines are often used to monitor viral growth in cell culture. Indicator cells contain an integrated expression cassettes consisting of a reporter gene such as Chloramphenicol Acetyl Transferase (CAT), beta-Galactosidase (beta-gal), Green Fluorescent Protein (GFP), Secreted Alkaline Phosphatase (SEAP) and others. The expression of the reporter gene is placed under the transcription control of the HIV promoter, the long terminal repeat (LTR). When the cells are infected with the virus, the viral protein Tat made in the cell will turn on the viral promoter which in turn will lead to the expression of the reporter gene, the function of which could be qualitatively and/or quantitatively determined. Monitoring viral growth using indicator cell lines offers the advantage of simplicity as opposed to direct evaluation of viral products like p24 and Reverse Transcriptase which is technically more challenging and expensive. Additionally, indicator cells also lend themselves for high-throughput screening to identify anti-viral agents. Indicator cell lines are also

expression. On viral infection, these cells synthesize SEAP which is secreted into the culture medium. The levels of SEAP in the spent medium are a measure of viral infection. A highly sensitive colorimetric assay is available for SEAP. Unlike several other reporter cell lines, these cell lines generate two different reports simultaneously in response to the viral infection. Importantly, measurement of both the reports is possible without terminating the experiment for analyzing the report gene expression. Secondly, these cell lines are the first to use an LTR derived form a primary Subtype-C viral isolate.

Experimental Scheme:

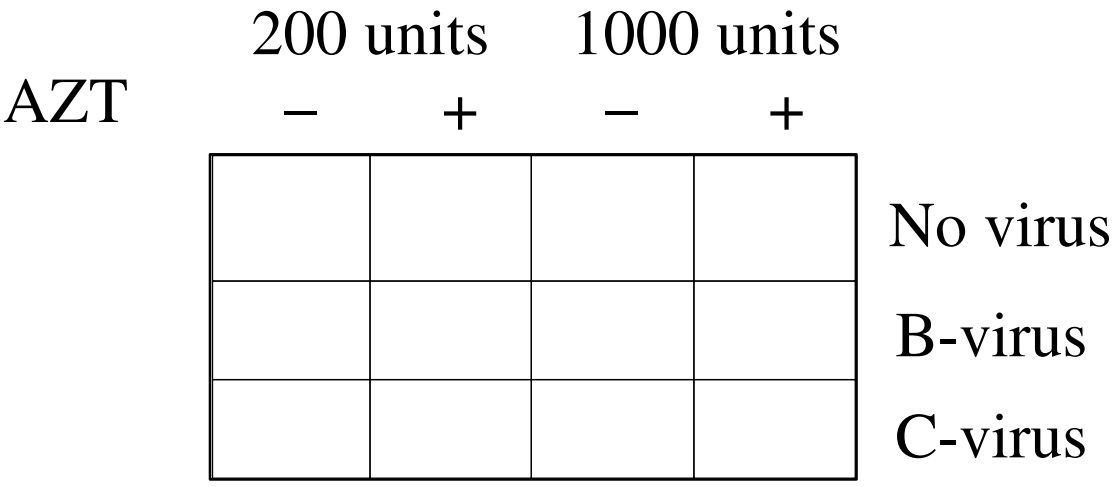
Day-1: Viral stocks of known titers will be added to the reporter cell lines. Anti-viral agents at different concentrations will also be included to monitor viral growth inhibition. Cells will be cultured in a CO_2 incubator.

Subsequent days: For the following five days, cells will be monitored for GFP

Experimental Protocol:

1) Count the number of cells and dispense $1x10^6$ cells/well in 0.5 ml of culture medium. Use a 12-well plate.

2) Add 50-100 $TCID_{50}$ (Tissue culture infective dose) of subtype-B and subtype-C viral preparation in 0.5 ml of culture medium.



Reagents:

1) 2x SEAP buffer
   2M Diethanolamine
   1 mM $MgCl_2$
   20 mM L-Homoarginine
   in sterile distilled water, store at 4°C

2) pNPP solution in SEAP buffer (1 mg/ml)

Protocol:

1) To 100 µl of 2x SEAP buffer in a well of a 96 well plate add up to 80 µl of the sample (make up the volume with sdw if necessary).
2) Add 20 µl of pNPP solution, mix well and incubate at 37°C.
3) Read the OD at different time points. Note that the reaction is read in real time as the enzymatic reaction is not terminated.
4) Read the OD at 405 nm.

Notes: The heat treatment of the sample is also intended to inactivate the endogenous alkaline phosphatases thus reducing the background values. Unlike SEAP, endogenous phosphatases are heat-labile. Additionally, homoarginine is an inhibitor of the endogenous phosphatases, however, this compound is not expected

Laboratory Session 5
Viral Load and CD4 measurements
Instructor: Dr. V. Ravi

Estimation of CD4 Cell Counts in human blood samples Using FACSCount

Principle of the assay:

This assay is based on the principle of flowcytometry and is used to enumerate CD4 and CD8 cells in samples of peripheral venous blood obtained from individuals. When whole human blood is added to reagent tubes containing CD4 and CD8 antibodies labeled with a fluorochrome, the reagents bind specifically to lymphocytes surface antigens. After an hour's incubation, a fixative solution is added to the reagent tubes, and the sample is subjected to analysis in the instrument. The samples whilst traversing through the flow cell come in contact with the laser light, which causes the fluorochrome labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells. The FACSCount machine is a dedicated system that can be used only for estimation of CD4 and CD8 cell counts. In this assay a convenient, ready to use reagent tube pair for estimation of both CD4 and CD8 cells are used: One tube determines the absolute number of helper/inducer T lymphocytes (CD4/CD3), and the other tube determines the absolute number of suppressor/cytotoxic T lymphocytes (CD8/CD3). Both tubes measure the absolute number of total T lymphocytes (CD3).

In addition to containing antibody reagent, the reagent tubes also contain a known number of fluorochrome integrated reference beads. These beads function as

PROCEDURE:

1. Preparing Controls:

a) Label the tabs of the two reagent tube pairs:
Pair one:
Label the CD4 (green top)- Zero
Label the CD8 (clear top)- Low
Pair two:
Label the CD4 (green top)- Medium
Label the CD8 (clear top) – high

b) Vortex the pairs upside down for 5minutes
c) Open the tubes with coring station
d) Mix normal whole blood and pipette 50ul into each of four reagent tubes
e) Cap the tubes and vortex
f) Incubation of tubes for one hour at 37°C
g) Add 50ul of fixative solution
h) Vortex the zero/low control bead pair and pipette 50ul of Zero control beads into the CD4 reagent tube labeled Zero
i) Pipette 50ul of low control beads into the CD8 reagent tube labeled low

TROUBLESHOOTING:

Troubleshooting includes pippeting errors, as well as instrument error codes and error messages that may appear on screen during operation. (refer to Becton Dickinson immunocytometry manual).

KEY REFERENCES:

1. *Becton Dickinson Immunocytometry Systems Manual*
2. *Young. N.L.,(1997) Clinical Field Evaluation of the FACS Count for Absolute CD3, CD3 CD4 and CD3 Cd8 Cell count Determinations in Thailand, Clin. Diag. Lab.Immunol, 4(6): 783-786.*

HIV 1-viral load assay using Real Time PCR (ABI PRISM 7000)

Principle of the Assay:

Viral load quantification is presently one of the methods for monitoring disease progression and assessing response to anti-retroviral therapy. Active replication of virus occurs in all clinical stages of infection. It is possible to detect and quantify virus throughout the course of HIV infection. Standardization of the Viral load assays is being done in our laboratory with the aid of a Real Time PCR (ABI PRISM) based on use of Taqman probes (Taqman Assay).

This Real Time PCR much like the conventional PCR, employs target specific primers. In addition a Taqman probe is also used. The Taqman probe is so designed that it binds to the target in between the two primers. This assay is based on the principle that Taq polymerase enzyme also has 5' nuclease activity. The AmpliTaq Gold DNA Polymerase used in this assay cleaves a taqman probe during PCR. The Taqman probe contains a reporter dye at the 5'end of the probe and a quencher dye at the 3'end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye which results in increased fluorescence of the reporter. Amplification of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Forster type energy transfer. During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'-3' nucleolytic activity of the AmpliTaq Gold DNA Polymerase

PROCEDURE:

a) RNA Extraction from HIV positive Plasma samples:

HIV RNA is extracted from HIV positive Plasma samples by using commercially available Columns (Auprep kit, Life technologies).

b) Reverse Transcription:

cDNA synthesis is carried out using High capacity cDNA Archive Kit (Applied Biosystems) as per manufacturers instructions. This kit contains reagents for reverse transcription of total RNA to cDNA**.**

Brief Method: Reaction Volume – 50µl (25µl RNA + 25µl RT mix)

a. Preparation of RT master mix:

	Per Reaction
10X RT Buffer	10µl
25X dNTP	4µl
10X Random primers	10µl
Multiscribe Reverse Transcriptase, 50U/µl	5µl
Nuclease free H2O	21µl

b. Aliquot 25µl RTmix followed by addition of 25 µl HIV RNA sample.

c. RT conditions are as follows: 25^{o}C for 10 mins

Flow Chart for running Real Time PCR:

Switch on the instrument 20 minutes before starting the reaction

↓

Preparation of PCR Master Mix (per reaction)

	Volume(µl)
2X Taqman Universal master mix	**12.5**
20X Primer probe mix	**1.25**
Double distilled water	**6.25**

After preparation of master mix the processed sample containing the viral RNA
(Template) is added as follows

Template Master mix

	Volume (µl)
PCR Master mix	**20**
Template	**5**

ABI PRISM 7000 system determines the absolute or relative quantity of a target nucleic acid sequence in a test sample by analyzing the cycle to cycle change in fluorescence signal as a result of amplification during PCR. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number of target nucleic acid.
After the run, the software processes the raw fluorescence data to produce threshold cycle (C_T) values for each sample. The software computes a standard curve from the C_T values of the diluted standards and extrapolates absolute quantities for the unknown samples based on their C_T values.

IMPORTANT TROUBLESHOOTING POINTS:

- Avoid Pipetting errors
- Avoid formation of air bubbles after addition of samples to the Reaction plate.

KEY REFERENCES:

1. ABI PRISM 7000 Sequence Detection System User Guide
2. Mackay.I.M., Arden. K., (2002) Real Time PCR in Virology, Nucleic.Acids.Res, 30(6):1292

Laboratory Session 6
Use of retroviral vectors for gene therapy
Instructor: Dr. Ganjam Kalpana

Note: An updated version of this protocol will be distributed at the session.

Production and infection of three-plasmid based vectors for the molecular analysis of HIV-1 Replication.

Reagents:

Plasmids: (Gift of Dr. Trono, Ref: Naldini et al. Science 1996)

(i) pMDG: (Expresing VSVg)

(ii) pHR'CMV-*lacZ*, or pHR'CMV-GFP (transducing vector expressing either *lacZ* or GFP).

(iii) pCMVΔR8.2 (Expressing Gag-Pol under CMV promoter)

Calcium Phosphate trasnfection Reagents:

Mammalian cell transfection kit (Specialty Media, Cat # s-001)

Reagents for Cell culture:

Plastic ware (Petriplates, pipettes, DMEM medium, FBS etc...)

Reagents for purification and concentration of the Virus:

Day 2:

(i) Change medium of 293T cells 1-3 hrs before transfection.

(ii) Prepare DNA for transfection. Preferably assemble inside the hood. For each 10 cm plate of cells, you need a total of 20-40 μg of three plasmid based vectors in a 2:1:1 ratio of transducing vector (pHR'-CMV-GFP), Gag-Pol vector (pCMV-ΔR8.2), and envelop vector (pMDG) respectively. Based on the number of plates to be transfected, assemble the DNA in eppendorf tubes. To this DNA add the specified amounts of TE, ultrapure ddWater, and CaCl2 as follows.

DNA	For one plate	Amount needed	For two plates	Amount needed
pHR'-CMV-GFP	10μg	Xμl	20μg	2Xμl

(iii) Prepare 1xHBS solution in 15 ml conical Falcon tubes as follows. You need 500 µl of 1x HBS per sample.

Reagent:	**For one plate**	**For two plates**
Ultrapure Water	440 µl	880 µl
10xHBS	50 µl	100 µl
NaOH	7.5 µl	15 µl
PO4	10µl	20 µl
Total	557.5 µl	1015 µl

(iv) Slowly add DNA mixture into the 1xHBS solution drop by drop while gently but constantly bubbling the air into 1xHBS solution. You could use one-ml pipette attached to a pipetter to bubble air. This will allow the

(iii) Filter the supernatant by passing through a low-protein binding syringe filter (Fisher cat # 09-740-37F).

(iv) Treat the Virus in the supernatants with 20U/ml of DNAse A (Roche) for 30 min at 37°C. DNase treatment is especially recommended during the infectivity studies using PCR aplification.

(v) Supplemented the virions with a final concentration of 50 mM HEPES, pH 7.4 (final concentration), aliquoted and stored at -80°C for further analysis. These virions are stable for extended periods of time. They retain most of the viability upon freeze-thaw.

(vi) If necessary, also collect the producer cells lysate for protein analysis. Briefly, scrape the cells in PBS, and pellet by low speed centrifugation. Resuspend each cell pellet in 200 µl of the RIPA buffer buffer (150mM

(iii) Carefully load the tubes into SW28Ti rotor and centrifuge at 15^{0}C for 3 hours at 25,000 rpm.

(iv) After the centrifugation, gently wash the pellet in 1x PBS to remove the remaining sucrose. It is very important to watch the pellet, as it is very loose and easily will get lost.

(v) Resuspended the pellet either in complete media for infection experiments, in PBS for other purposes. It is very important to vigorously resuspend the pellet by pipetting up and down at least a dozen time.

(vi) Supplement the resuspended pellet with 50 mM HEPES, pH 7.4 (final concentration), and store at -70°C.

(vii) For the purposes of immunoblot analysis, resuspend the virion pellets in the virion lysis buffer (25 mM Tris-Cl, pH 7.5, 50 mM KCl, 0.025% Triton

(v) Transfer virion-containing fractions were then transferred into fresh centrifuge tubes, and fill the tubes to the top with TNE buffer (10 mM Tris HCL [pH 7.2], 100 mM NaCl, and 1 mM EDTA).

(vi) Centrifuged at in SW28Ti rotor at 25,000 rpm for 2 hours.

(vii) Collect the purified concentrated virus pellets by resuspending in the lysis buffer and 2X sample buffer, and stored at -70°C.

To determine the infectious titers of the three-plasmids based viral vectors harboring reporter gene.

To determine the infectivity of the HIV-1 GFP single-cycle infectious virus particles produced in the studied cell lines, virus-containing culture supernatants normalized for levels of p24 antigen are used to infect target cells. 293T cells or Hela cells can be used as three-plasmid based vectors are peudotyped with VSVg.

Day 7:

Percentage of infected cells was determined by calculating the fraction of GFP-positive cells in a FACSort analyzer. The number of foci formed can also be calculated visually and then converting to foci per plate.

Percentages of infected cells resulting from infections with the serial dilutions of the virus are then converted to percentage per 1ml of raw culture supernatant, and the averages are calculated. These numbers are used to calculate the percent of infected cells per ng amount of virus p24.

Laboratory Session 7
Heteroduplex mobility shift assays
Lab Session Leader: Dr. Udaykumar Ranga

Note: Printed version of the power point presentation of Dr. Ranga's talk is provided as part of a separate handout. The CD will include this presentation.

HMA (Heteroduplex Mobility Assay)
for Genetic Characterization of HIV-1

HMA is a method to characterize sequence differences between two or more fragments of DNA using gel electrophoresis. A mixture of double stranded DNA molecules is denatured by heating and the single strands are allowed to come together by cooling. When the single strands of DNA from two different molecules come together, or anneal, the presence of mismatches between them leads to the formation of "bubbles" in the heteroduplexes. These bubbles retard the migration of heteroduplexes, and in proportion to the extent of sequence differences, to help characterize the nature of sequence differences.

High levels of genetic heterogeneity in HIV-1 is the basis for its classification into three major groups and more than 11 subtypes. It is necessary to identify the genetic subtype of HIV-1 at the individual- and population-levels to study molecular epidemiology. Even though different HIV-1 subtypes show distinct geographic distributions, an increasing prevalence of multiple subtypes is being observed in a number of countries. The presence of multiple subtypes in a region has also led to the formation of new inter-subtype recombinant viruses. Therefore, monitoring the prevalence of different subtypes in an epidemic is essential for understanding the epidemic. Because of the high levels of genetic heterogeneity, vaccine candidates based on one subtype are not expected to be effective for other subtypes and the evolution of recombinant viruses further complicates the issue. In addition, HIV-1 subtypes may also differ in their virulence affecting the spread, and in the response

HMA for Subtyping HIV-1 can be spread over four days as below:

Day 1: sample preparation and DNA extraction;

Day 2: PCR amplification and agarose gel electrophoresis

(If necessary, days 1 and 2 (sample preparation and PCR amplification) can be combined in to one day);

Day 3: Polyacrylamide gel electrophoresis for HMA

Day 4: Analysis and interpretation of HMA

DNA Extraction and PCR (depends on whether we would like to extract DNA from fresh blood or use previously subtyped viruses for the unknowns):

The best way to train candidates is to use whole blood from HIV infected individuals. This will train the individual for all the steps starting from DNA extraction to running HMA gels and to interpreting them. This will require fresh whole blood from one or more HIV-infected individuals to be made available. If using HIV-infected whole blood is not possible, I would suggest using frozen DNA extracted from ficoll-hypaque purified PBMC or from whole blood.

For extracting DNA from blood, we can either purify PBMC over ficoll-hypaque and extract DNA or extract the DNA from the whole blood using whole blood DNA extraction kit. I have used puregene kit from Gentra systems and it works well (http://www.gentra.com/products.asp?product_family_ID=1&productmenu_ID=5)

If the candidates are going to be trained in purifying PBMC over ficoll-hypaque, then we will need ficoll-hypaque solution, PBS, plasticware etc., (This is not my preference). If this is the way we are going, then I will provide the catalog numbers etc.,

Lysis buffer
10 mM Tris HCl, pH 8.3

Reagents/Equipments needed:

PCR amplification kit
10X Taq buffer, Taq, MgCl2, RNase-free water (Invitrogen)
dNTP mix

10X Heteroduplex Annealing Buffer
1000 mM NaCl
100 mM Tris HCl, pH 7.8
20 mM EDTA

10X TBE Gel Electrophoresis Buffer
880 mM Tris-borate, pH 8.0
890 mM Boric Acid
20 mM EDTA
One liter of 10X stock is prepared by dissolving in deionized H_2O: 108 g of Tris base, 55 g of Boric acid and 40 ml of 0.5 M EDTA.

10X TAE gel electrophoresis buffer:
400 mM Tris-acetate
10 mM EDTA
One liter of 10X stock is prepared by dissolving in deionized H2O: 48.8 g of Tris

Miscellaneous Items/Reagents needed:
Thermal Cycler
UV-transilluminator
Photodocumentation system
Microwave
24-well or higher microcentrifuge
Gel electrophoresis power supply
Thin wall PCR reaction tube strips
Ice buckets
Roll of parafilm
Roll of plastic film (household use variety)
Kimwipe Low lint wipers (Fisher Cat # 06-666C, pack of 15 boxes for $170 or $13.80 per box individually)
Plastic backed Absorbent surface liners Fisher 18”x 50’ Cat 14-127-49 (2 or 4 rolls)
Agarose (Electrophoresis grade) 500 grams
Boiling water bath
Eppendorf microcentrifuge tubes,
50 ml centrifuge tubes
1, 5, 10, 25 ml pipettes
battery operated pipettors
p20, p200 and p1000 pipette sets (how many?)
p20, p200 and p1000 aerosol-barrier pipet tips
Gel-loading tips (Fisher Cat # 02-707-173 $99 for a pack of 1020
250 ml Conical flasks for heating agarose gels and for mixing polyacrylamide gels

Useful links
(Compiled by Drs. Oyekanmi Nash and Dibyakanti Mandal)

1. For HIV Researchers

http://www.unaids.org
The Joint United Nations Program on HIV/AIDS, UNAIDS, is the main advocate for global action on the epidemic. It leads, strengthens and supports an expanded response aimed at preventing transmission of HIV, providing care and support, reducing the vulnerability of individuals and communities to HIV/AIDS, and alleviating the impact of the epidemic.

http://www.who.int/hiv/en
WHO works within the family of UNAIDS Cosponsors to facilitate multisectoral efforts within the United Nations system. As part of its broad health-sector mandate in HIV/AIDS, it specifically serves as the convening agency within the United Nations system for HIV/AIDS treatment, care and support as well as for preventing the mother-to-child transmission of HIV.

http://hiv-web.lanl.gov
The HIV databases contain data on HIV genetic sequences, immunological epitopes, drug resistance-associated mutations, and vaccine trials. The website also gives access to a large number of tools that can be used to analyze these data. This project is funded by the Division of AIDS of the National Institute of Allergy and Infectious

reagents were provided. US scientists obtaining AIDS reagents from the program work primarily in academic settings. There are also users from industry and the Federal Government.

http://www.AmFAR.com
The American foundation for AIDS Research (amfAR) is the nation's leading non profit organization dedicated to the support of AIDS Research, AIDS prevention, treatment education and the advocacy of AIDS related public policy.

http://www.pedaids.org
The Foundation creates a future of hope for children and families worldwide by eradicating pediatric AIDS, providing care and treatment to people with HIV/AIDS, and accelerating the discovery of new treatments for other serious and life-threatening pediatric illnesses.

http://www.cmmg.biosci.wayne.Edu
Comprehensive website on global epidemic of HIV-1 especially related to African continent.

http://www.bioafrica.net/subtype
This link is useful for HIV researcher regarding detailed information about the subtype distribution in Africa.

http://hivinsite.ucsf.edu

of 2002, we organised two interdisciplinary regional training workshops on HIV/AIDS and Human Rights in the Asian and African Region. ...

http://www.neaetc.org/
The New England AIDS Education & Training Center
To increase the number of health care providers effectively trained to counsel, diagnose, treat and manage the care of individuals with HIV infection and to assist in the prevention of high risk behavior which may lead to infection. Didactic presentations, participatory workshops, clinical consultation.

http://www.usaid.gov/locations/europe_eurasia/car/briefers/hivaids_prevention.html
USAID Europe and Eurasia: HIV/AIDS Prevention
USAID/CAR has developed a strategic program designed to prevent drug use among vulnerable youth and control the HIV/AIDS epidemic among high-risk groups, such as drug users and sex workers. The strategy is designed for all five countries with a main focus on Uzbekistan, Tajikistan, and Kazakhstan.

http;//www.ucsf.edu/hivcntr/Training/Workshops.html
NCCC : National HIV/AIDS Clinicians' Consultation Center
AIDS Education and Training Centers The NCCC presents its National AETC Faculty Development Workshop for AETC Consultants and Pharmacists each summer in San Francisco. The Workshop is targeted to AETC consultants (including MDs, RNs, NPs,

http://www.worldywca.org/aids/aids_policies.html
HIV/AIDS World YWCA Policy Statements
The World YWCA has set consistent policy over the years to make HIV/AIDS a priority for the movement as defined within the following statements:

http://www.jhuccp.org/training/Workshop/Workshop.htm
JHU- Workshops
Training Division workshops focus on health communication and advocacy, communication and advocacy for youth programs, leadership and management, and interpersonal communication and counseling.Three workshops are held annually in the US in a ... communication and advocacy strategies for HIV/AIDS, using "SCOPE ... Training of Trainers of NGOs in Advocacy and ...

http://www.actoronto.org/website/home.nsf/pages/youthedopps
Workshops, Training Sessions, and Partnership Opportunities for ...
Are you interested in having a workshop for youth in your shelter, community centre, school, work place, youth organization, peer group, etc? Would you like to learn more about educating youth on HIV/AIDS, safer sex, and healthy sexuality? Would you like safer sex and harm reduction information and materials targeting youth for your community centre or youth organization? We offer workshops and education opportunities for youth and staff who work with youth in many areas. Our interactive workshops can be tailored to fit your needs

communities to better address healthcare priorities, while improving productivity and quality of care.

http://www.aidsnews.org.il/abouteng.htm
The Jerusalem Aids Project
JAIP is an independent, International NGO, which gained wide experience in the last 5 years in the area of professional training in HIV/AIDS both in the Middle East and in developing countries in Asia and Latin America. Teams of experts from the project w ere involved in 35 major community-based AIDS Education interventions in 21 countries, in addition to the work done on a national scale in Israel. The JAIP training workshops are conducted within a total number of 40 hours ... It covers the following topics: HIV/AIDS Epidemiology in youth, clinical aspects ...

2. HIV/AIDS Training workshops-related sites

HIV/AIDS Surveillance Data Base
The *HIV/AIDS Surveillance Data Base* was developed and is maintained by the Health Studies Branch, International Programs Center (IPC), Population Division, U.S. Bureau of the Census, with funds from the U.S. Agency for International Development. It is a compilation of information from those studies appearing in the medical and scientific literature, presented at international conferences, and appearing in the press.
www.census.gov/ipc/www/hivaidsd.html

PAs, and PharmDs), medical directors, evaluators and other AETC faculty interested in current clinical consultation topics.
www.ucsf.edu/hivcntr/Training/Workshops.html

NASW HIV/AIDS Spectrum
Working in collaboration with NASW chapters, federal and state agencies, national and state associations, universities, and community-based organizations, the *HIV/AIDS Spectrum: Mental Health Training and Education of Social Workers Project* offers education and training to mental health care providers on the mental health aspects of living with HIV/AIDS.
www.socialworkers.org/practice/hiv_aids/spectrum.asp

People living with HIV/AIDS
The International HIV/AIDS Alliance works with communities to prevent the spread of HIV,support and care for those infected and ease the impact of HIV on families and communities. The Alliance recognizes and promotes the involvement of people living with HIV/AIDS (PLHA) as a central strategy to ensure stronger community responses to the epidemic and more appropriate public policy
www.aidsmap.com/web/pb4/eng/ F00CEDCE-8C0F-11D5-8D07-00508B9ACEB1.htm

Pacific AIDS Education and Training Center
The Pacific AIDS Education and Training Center at the Keck School of Medicine of the University of Southern California offers educational programs for physicians,

HIV/AIDS, Hepatitis C and Sexual Health Website: Training and ...
Step Out Facilitator Training provides the skills necessary to facilitate QuAC's Step Out workshops for young gay and bisexual men. Step Out workshops operate with bisexual and gay men aged 16 to 26 and run periodically. The training provides the skills regarding; HIV and other sexual transmitted infections, coming out, self esteem, family and religion and the gay community.
www.health.qld.gov.au/sexhealth/training/gaymen02.shtml

JHU- Make A Difference HIV/AIDS
"Make a Difference": A Strategic and Multisectoral Approach to HIV/AIDS Communication Workshop
Baltimore, Maryland, USA April 18- May 6, 2005
A three-week workshop for US and International decision-makers, administrators, health educators, program officers, managers and donor agency field staff working on HIV/AIDS.
www.jhuccp.org/training/Workshop/MakeADifference.htm

Interdisciplinary School of Health Sciences, University of Pune The Ford Foundation has sanctioned a capacity building programme which offers fellowships, small grant projects, national level meetings, workshops and number of training programmes to develop skills and generate research in the Social and Behavioral aspects related to Sexuality, Reproductive Health, HIV/AIDS.
www.unipune.ernet.in/dept/shs/social%20behaviour.html

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Program at a glance

31st July	-	Laboratory Workshop Day 1 *JNCASR* *Lab sessions: Drug susceptibility measurement, ELISPOT, Detection of M. tuberculosis via reporter phages, Reporter cell lines for HIV.*
1st August	-	Laboratory Workshop Day 2 *AM Session: NIMHANS* Lab sessions: Viral load and CD4 measurements *PM Session: JNCASR* *Lab sessions: ELISPOT assay, Use of lentiviral vectors for gene therapy*
2nd August	-	Symposium Inauguration - *JNCASR* *Plenary Session* *Symposium Session I – HIV/AIDS* *Symposium Session II – Prevention, Vaccines and Antiretroviral Strategies* Cultural Program & Banquet