Human Immunodeficiency Virus Protein-Protein Interactions: Developing High Throughput Screens for Novel Drug Targets.

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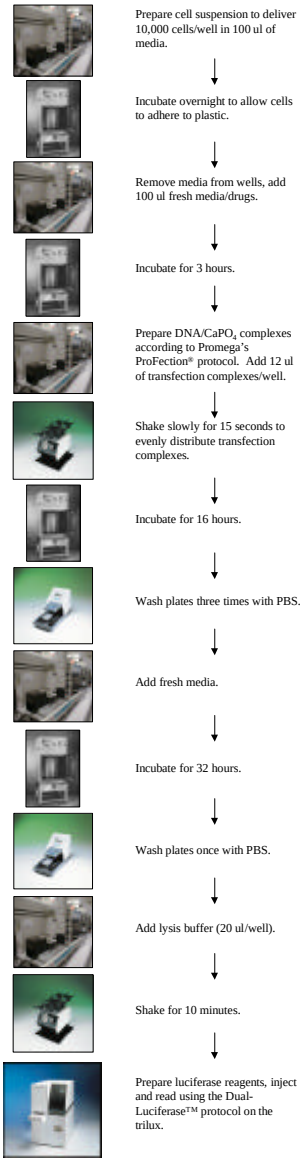
Abstract

Development of drug resistance to current therapies for human immunodeficiency virus type 1 (HIV-1) infection has lead to the need for additional strategies for drug treatment. The HIV-1 regulatory and structural proteins represent drug targets that have not yet been exploited. The Serquest HTS group has developed an extensive panel of assays to test for inhibitors of protein-protein interactions between HIV genes and their corresponding viral and cellular targets. This system utilizes the Promega Dual-Luciferase™ Reporter Assay System and has been adapted to a Beckman-Coulter Core robotics platform. Finding inhibitors of the interactions of these proteins with other viral and/or cellular proteins is a novel approach to HIV-1 drug discovery. With viral resistance limiting current therapies, such compounds could prove useful in treating HIV infected individuals.

Limitations of Current HIV Therapies

• Of the 15 viral proteins only two, RT and PR, are targeted by current therapies.
•Drug resistance will always be a problem when targeting these enzymes.
• Will never be able to eradicate virus from patients using these therapies alone (>60 years).
• These therapies do not target the proteins that cause the pathogenic effects of the virus.

Use of a Beckman Coulter Core Platform to run the Promega Checkmate™ Mammalian Two-Hybrid System



Prepare cell suspension to deliver 10,000 cells/well in 100 ul of media.

Incubate overnight to allow cells to adhere to plastic.

Remove media from wells, add 100 ul fresh media/drugs.

Incubate for 3 hours.

Prepare DNA/$CaPO_4$ complexes according to Promega's ProFection® protocol. Add 12 ul of transfection complexes/well.

Shake slowly for 15 seconds to evenly distribute transfection complexes.

Incubate for 16 hours.

Wash plates three times with PBS.

Add fresh media.

Incubate for 32 hours.

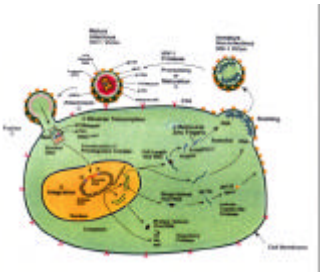
Wash plates once with PBS.

Add lysis buffer (20 ul/well).

Shake for 10 minutes.

Prepare luciferase reagents, inject and read using the Dual-Luciferase™ protocol on the trilux.

Human Immunodeficiency Virus Life Cycle



Potential Viral Targets

•Enzymes: Reverse Transcriptase (RT)/RNaseH, Protease (PR), Integrase (IN)
•Structural Proteins: Matrix (MA), Capsid (CA), Nucleocapsid (NC), pr55-Gag, p6
•Glycoproteins: gp120, gp41
•Regulatory Proteins: Tat, Rev, Nef, Vif, Vpu, Vpr
•Others: Ribosomal Frameshifting, packaging signals, etc.

Tat

Background
•Transcriptional regulator of viral gene expression
•Mediates high-level expression of all viral genes
•Repressor of certain cellular promotors

Functions
•Binding to Cyclin T1 and TAR
–Actively binds to Cyclin T1 and recruits it to the transactivation response element (TAR) RNA
–Cyclin T1 then binds CDK9 which phosphorylates RNA Pol II, allowing for processive elongation of HIV-1 RNA
•Transactivation through p300 and CREB-binding protein (CBP)
–Both p300 and CBP have histone acetyltransferase activity
–Tat binds and recruits these proteins to the integrated viral LTR
–p300 and CBP acetylate histones, weakening the histone-DNA interactions, thereby relieving the repressive effects of the chromatin scaffold on the LTR

Assay Development
•Inhibition of Tat-p300 and Tat-CBP interactions using two hybrid system
•Inhibition of Tat binding to LTR
•Inhibition of Tat-Cyclin T1 interaction using two hybrid system
•Inhibition of Tat binding to TAR

Vpu

Background
•Integral membrane protein
•unique to HIV/SIVcpz viruses
•facilitates viral maturation and release
•destabilizes intracellular gp160/CD4 complexes
•Induces intracellular degradation of CD4

Functions
•CD4 degradation and gp160 liberation
–gp160 binds CD4 in the ER and prevents its transport to the cell surface
–Vpu then binds CD4, facilitating the release of gp160, while still retaining CD4 within the cell
–Vpu then targets CD4 for degradation by binding to hßTrCp, a component of the ubiquitin mediated protein degradation pathway
•Virus particle release
–Vpu binds a cellular protein, UBP, that helps facilitate virus particle assembly and release
–Both MA and CA have been described to be involved with this process
–This suggests either a direct interaction of Vpu with Gag, or an indirect interaction through UBP

Assay Development
•Inhibition of gp160-CD4, Vpu-CD4, and Vpu- hßTrCp interactions using two hybrid system
•Inhibition of Vpu-Gag and Vpu- UBP interactions using two hybrid system

Nef

Background
•Myristylated cytoplasmic protein
•required for *in vivo* viral replication and pathogenesis
•Enhances viral replication
•Increases efficiency of reverse transcription
•Enhances serine phosphorylation of MA
•Involved in control of syncytia formation
•Enhances virion infectivity
•Interferes with host cell signaling pathways through interactions with many cellular signaling proteins
•Downregulates levels of CD4, MHC class I, and envelope on the cell surface

Functions
•CD4 and MHC class I downregulation
–Important to prevent superinfection of cells and to evade the immune system
–Both involve Nef binding to clathrin adaptor complexes
–Nef directly interacts with CD4 cytoplasmic tail
–MHC class I mechanism is less understood
–Nef directly binds additional cellular proteins to mediate CD4 downregulation: NBP1, hACTE-III, NAF1
•Interference with and regulation of host cell signaling pathways
–Nef interacts with numerous cellular signaling proteins: Hck, Lck, CD4, MAPK, c-Raf1 kinase, p62/PAK/Serine kinase
–Cell dysregulation is responsible for much of the pathogenesis caused by HIV
–Allows the virus to take control of the cell

Assay Development
•Inhibition of above described protein-protein interactions using two hybrid system
•PLAP flow cytometry assay

Vif

Background
•Virus infectivity factor
•Functions late in the replication cycle, at time of virus particle assembly
•Enhances infectivity of virus particles released
•Late function of Vif has an effect on initiation of reverse transcription in subsequent rounds of replication
•Regulates Protease activity
•Requires phosphorylation for it to be functional

Functions
•Regulation of Protease activity
–Vif interacts with HIV-1 PR to regulate its activity
–This is necessary to prevent PR from cleaving Gag and Gag-Pol polyproteins at the wrong stage of the replication cycle
–If these proteins are cleaved too early, some of there smaller cleavage products will not get incorporated into virus particles
•Inhibition of Cellular Anti-HIV-1 Protein
–Recent studies have described a natural cellular anti-HIV-1 phenotype expressed by some cells
–Vif counteracts this phenotype and is required for HIV-1 growth in these cells
–Explains the necessity for Vif during HIV-1 replication in vivo
–Represents a good future target once mechanism is worked out

Assay Development
•Inhibition of Vif-PR interaction using two hybrid system
•Inhibition of Vif to unknown cellular protein interaction using two hybrid system

Vpr

Background
•incorporated into viral particle through interactions with Gag
•important for nuclear import of preintegration complex
•important for efficient viral replication in natural target cells
•induces cell cycle arrest at G2
•reduces mutation rate of HIV during replication
•stimulatory effect on transcription from LTR

Functions
•Nuclear import of preintegration complex
–Vpr stabilizes the interaction of karyopherinα/β heterodimers with the nuclear localization signal of MA
–This allows the karyopherins to mediate nuclear import
–Vpr also interacts directly with nucleoporins, the proteins that make up the nuclear pore complexes and regulate the flow of molecules in and out of the nucleus
•Cell cycle arrest
–cdc25c is a phosphatase that regulates the cell cycle
–For entry into mitosis, cdc25c must activate the cyclin B-p34cdc2 kinase complex by removing phosphates from p34cdc2
–Growth arrest occurs when Vpr binds to cdc25c, preventing it from activating this complex
–Binding of Vpr to HHR23A and mov34 also believed to be involved

Assay Development
•Inhibition of Vpr-karyopherin, Vpr-MA, and Vpr-nucleoporin interactions using two hybrid system
•Inhibition of Vpr-cdc25c, Vpr-HHR23A, and Vpr-mov34 interactions using two hybrid system
•Flow cytometry based system to test for compounds that disrupt the Vpr cell cycle arrest function

Rev

Background
•Posttranscriptional regulator of viral gene expression
•facilitates nuclear export, stability, and translation of viral messages that contain the Rev Responsive Element (RRE)

Functions
•Protection of Genomic RNA from splicing
–In the absence of Rev, all viral RNA is spliced prior to export from the nucleus
–Through binding to the RRE, oligomeric Rev protects HIV-1 genomic RNA from being spliced
•Nuclear export of viral RNA
–Rev is imported to the nucleus through binding to Importin-ß
–Once bound to viral RNA, Rev mediates its nuclear export
–This is accomplished through Rev binding to hCrm-1, a host cell nuclear export factor

Assay Development
•Inhibition of Rev-Rev interaction (oligomerization) using two hybrid system
•Inhibition of Rev binding to RRE
•Inhibition of Rev-Importin-ß and Rev-hCrm-1 interactions using two hybrid system

Additional Assay Development

Integrase
•Complementation assay
•Intracellular integration
•Oligo-based assay
•Two Hybrid system:
–IN binding to IN (multimerization)
–IN binding to Ini1 (selective integration)
–IN binding to RT (initiation of RT)
–IN binding to UDG (UDG incorporation into virions)
–IN binding to Karyopherins (High MOI nuclear import)

Structural Proteins
•Gag binding to Vpr (Vpr incorporation into virus)
•Gag binding to Vif (Vif incorporation into virus)
•Gag binding to PR (regulation of PR activity)
•Gag binding to Actin (virus particle assembly)
•Gag binding to Cyclophilin A (CA core assembly)
•MA binding to MA
•MA binding to karyopherins (nuclear import)
•MA binding to Vpr (nuclear import)
•MA binding to gp41 (incorporation into virus)
•MA binding to HO3 (enhances infectivity of virus particles released)
•CA binding to CA
•CA binding to Cyclophilin A (disassembly of the viral core)
•NC binding to psi site
•NC binding to RT
•NC binding to Vpr (Vpr incorporation into virus)
•NC binding to Actin (virus assembly)