Viral Phenotypic Resistance Assays

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1 Introduction

The emergence of the human immunodeficiency virus (HIV) pandemic in the early 1980s led to a marked escalation in virology research. A rapidly expanding knowledge base percolated not only within the HIV field but also in that of other viral diseases. The identification of drug targets in these viruses led to the development and approval of antiviral agents. However, especially for HIV, it quickly became apparent that the use of these agents could select for drug-resistant viruses. The need for assays to identify resistant strains and to guide physicians in treatment decisions was urgent. Today, the availability of numerous antiretroviral agents for HIV therapy, combined with assays to guide their use, allows the selection of combination regimens that can effectively suppress HIV replication for many years. The vast amount of experience gained over many years of HIV drug development and clinical research notably hastened more recent hepatitis C virus (HCV) drug development efforts. Combination drug regimens for HCV that include one or more direct-acting antiviral agents to different targets have been evaluated rapidly and optimized to minimize the emergence of resistance-associated variants and to promote viral clearance.

Phenotypic susceptibility assays are used for some viruses in a clinical setting. For HIV, they can help with the selection of the most active drug regimen for an individual's viral population. They are also employed in research studies, drug discovery, and preclinical and clinical stages of drug development, for example, to characterize resistance and cross-resistance patterns for new drugs and to establish correlations between discrete genotypic changes and drug susceptibility.

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Viral phenotypic susceptibility assays are designed to determine the observable susceptibility or resistance of a virus to an antiviral agent. Numerous types of assay have been described including classic plaque assays and more recent recombinant virus assays (RVAs). Susceptibility or resistance to an antiviral agent in cell culture is often reported as the concentration of antiviral agent that inhibits viral replication by 50 or 90% (IC_{50} or IC_{90}, respectively). The IC_{50} or IC_{90} is typically compared to that of a control or reference virus that is assumed to be drug sensitive, and the results are expressed as a ratio (often referred to as fold change or resistance index) of the experimental virus versus the control (e.g., IC_{50} experimental virus/IC_{50} control virus).

This chapter reviews the major phenotypic antiviral susceptibility assays, with a focus on HIV- and HCV-related assays. The use of intact virus assays, the development and clinical applications of recombinant virus assays for HIV drug resistance, replication capacity and coreceptor tropism determination, the use of HCV replicon assays for drug development, and the status of phenotypic assays for other viruses including HBV, CMV, HSV, and influenza virus are discussed.

2 Intact Virus Susceptibility Assays

2.1 Plaque Assays

Plaque assays were originally developed to study bacteriophages in the early twentieth century [1]. In the early 1950s, the assay was adapted for poliovirus by Dulbecco and Vogt [2–4] and catapulted animal virology forward. Plaque assays are based upon the principle that a single virus particle infecting a single cell in a monolayer culture will lead to a local area of cytopathology (a "plaque") after subsequent infection of adjacent cells when the culture is overlaid with a semisolid nutrient medium to prevent long-range secondary infection through diffusion. The amount of time required for plaque formation depends on the type of virus, cells, and growth conditions. Plaques are identified visually, often by staining the remaining viable cells. The plaques then appear

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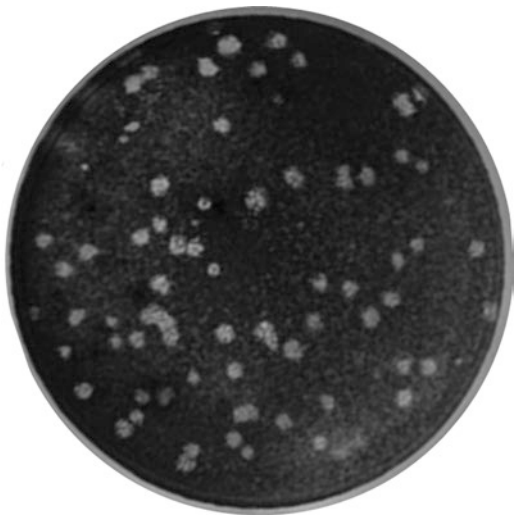

Fig. 83.1 Plaque assay. Crystal violet stained microtiter plate well showing HSV plaques in Vero cells (Image *source*: http://en.wikipedia.org/wiki/File:Plaque_assay_macro.jpg)

as clear circles in a stained monolayer of cells (Fig. 83.1). Alternatively, the monolayer can be stained with an antibody specific for viral antigens and the plaques (or foci) identified by colorimetric or fluorescence detection methods. The number of "plaque-forming units" (pfu) or "focus-forming units" (ffu) in a given volume is a measure of the infectious virus titer in a sample.

Plaque assays can be used to measure drug susceptibility. For example, serial dilutions of an antiviral agent can be added to the growth medium of both control and test virus infections. A dose-response curve (pfu/mL versus drug concentration) can then be generated, and the IC_{50} or IC_{90}, or change in IC_{50} or IC_{90} relative to control, can be determined. These types of "plaque reduction assays" have been utilized to measure drug susceptibility of many viruses, including influenza [5], herpes simplex (HSV) [6], cytomegalovirus (CMV) [7], varicella zoster virus (VZV) [8], and HIV-1 [9] (see below). One advantage of plaque assays over some other types of infectivity assays is that they can provide a visual assessment of viral fitness, as reflected by the size of the plaque. In addition, the presence of a low-level minority species of resistant virus can be detected by virtue of in vitro selection that can occur during a culture-based assay.

2.2 Virus Yield or Antigen Expression Assays

As an alternative to plaque reduction assays, virus released into the liquid medium of an infected cell culture in the absence and presence of antivirals can be measured by various techniques and used to quantitate antiviral susceptibility. The quantity of virus in the medium can be determined based on infectivity (e.g., by plaque assay or 50 % infectious dose ($TCID_{50}$) titration), viral antigen production (e.g., by ELISA), cytopathic effect (CPE), or viral nucleic acid production. Virus yield reduction assays have been used to measure drug susceptibility of several viruses including HIV [10], HSV [11–13], influenza virus [5, 14], and CMV [12, 15, 16], as detailed below.

2.3 Limitations of Intact Virus Assays

Plaque reduction and viral yield reduction assays are labor intensive, and some have limited precision, making them difficult to perform on a large scale for routine clinical use. The assays use replication-competent virus, which may undergo multiple rounds of infection during the assay. Thus for viruses that replicate with a high error rate, the virus tested in the assay could have acquired altered characteristics compared to those of the original virus sample. Additional limitations of intact virus assays include biosafety concerns that can make large-scale operations involving handling of infectious virus stocks a logistical obstacle. The ability to recover infectious virus from clinical specimens is not always reliable and is dependent on titer and fitness, which can vary considerably. Finally, some viruses do not form visible plaques, and others lack an in vitro cell culture system (or a system amenable to routine use) for clinical isolates and thus cannot be studied using plaque or other cell-based assays that rely on infection by intact viruses derived from clinical material.

3 Phenotypic Drug Susceptibility Assays for HIV-1

3.1 Plaque Reduction Assays

Initial measurements of HIV drug susceptibility, including the first description of zidovudine-resistant HIV-1 from infected individuals [9], were made using a plaque reduction assay in HeLa cells engineered to express the CD4 receptor [17]. Plaques, or foci, of infected cells could be identified and counted based on the propensity of the infected cells to fuse and form multinucleated syncytia; reduction in plaque/focus number in the presence of drug was used to derive IC_{50} values. Detection of infected cells was simplified by introduction of a β-galactosidase reporter gene under the control of the HIV-1 LTR [18]. Initially, these assays only generated plaques or foci with syncytium-inducing (SI) virus, since HeLa cells naturally express the CXCR4 coreceptor, but not CCR5 (see Sect. 3.5). Artificial expression of CCR5 in HeLa/CD4 cells, or other cell lines, overcame this obstacle [19–22].

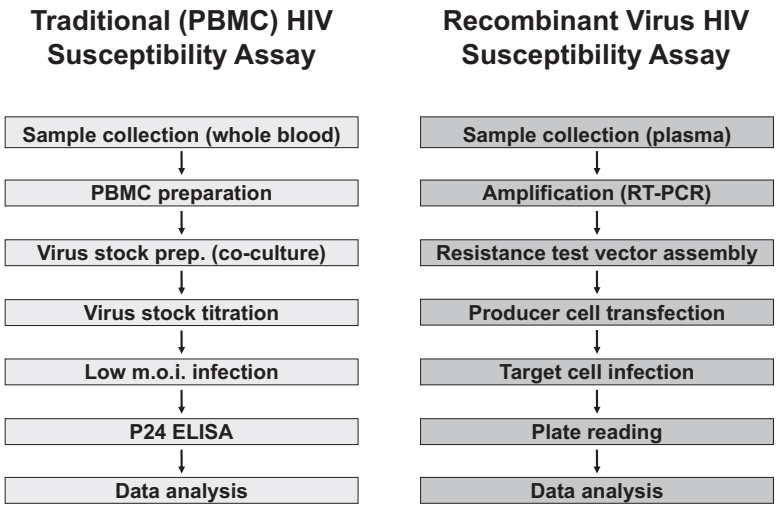

Fig. 83.2 Comparison of the process flow for intact virus (PBMC) and recombinant virus (PhenoSense HIV) assays

3.2 Peripheral Blood Mononuclear Cell-Based Assays

In the early 1990s, an alternative HIV phenotypic assay method was developed in which peripheral blood mononuclear cells (PBMCs) from an HIV-infected individual were co-cultured with phytohemagglutinin (PHA)-stimulated PBMCs from a seronegative donor [10] (Fig. 83.2). After approximately 7 days, the supernatant of the culture was collected as the viral stock and was subsequently titrated (based on p24 antigen production) on more PHA-stimulated donor PBMCs for an additional 7 days. An appropriate dilution of the viral stock was then added to PHA-stimulated donor PBMCs and grown for a further 7 days in the absence and presence of an antiretroviral agent. The supernatant was harvested and p24 antigen measured by an ELISA to quantitate virus production and generate susceptibility curves and IC_{50} or IC_{90} values. While this assay was standardized and provided useful phenotypic drug susceptibility/resistance data, it was cumbersome, imprecise, and slow. In addition, it is possible that the HIV stock derived from latent provirus in infected PBMCs does not reflect the strains circulating in the plasma.

3.3 Recombinant Virus Assays

The first recombinant virus assay for HIV generated viable virus by homologous recombination of a reverse transcriptase (RT)-deleted SI viral clone with a PCR-derived pool of RT sequences derived from proviral DNA samples [23]. Recombinant, replication-competent virus was amplified in a T-cell line and the virus harvested after 8–10 days, followed by virus titration and determination of drug susceptibility in a HeLa CD4+ cell foci reduction assay [23] or cell killing assay using a colorimetric readout [24, 25]. This assay represented a major step forward as it eliminated the need for donor PBMC cultures, thus standardizing viral stock production. Additionally it reduced the potential for the selection of virus stocks in culture that might differ from those represented in original sample due to the selective effects of different HIV gene products, particularly envelope. However, the use of proviral DNA may not fully reflect the circulating replication-competent virus, and the turnaround time for these assays (3–4 weeks) was still significant. This assay was later modified to measure HIV protease (PR) inhibitor susceptibility and to amplify sequences from plasma viral RNA instead of proviral DNA [26]. The assay was commercialized by Virco (Antivirogram®) in 1998 but discontinued for routine clinical use in 2010.

Significant advances that facilitated the use of phenotypic assays for routine clinical use occurred in the late 1990s. Both VIRalliance and ViroLogic (now Monogram Biosciences Inc.) developed and commercialized more rapid HIV phenotypic assays to measure resistance to antiviral drugs. The VIRalliance assay (Phenoscript™) [27] involves separate amplification of the gag-PR and the RT regions of HIV from RNA extracted from plasma samples. Each PCR product is then separately co-transfected into HeLa cells along with a proprietary plasmid vector. Infections are limited to a single cycle to ensure that the recombinant virus accurately reflects the amplified region from a clinical sample. Single-cycle infection is achieved by the deletion of the envelope region from the vector; recombinant virus is pseudotyped with the G-protein of the vesicular stomatitis virus (VSV-G). For testing of protease inhibitors, the transfected viral producer cells are incubated in the presence of serial dilutions of drug. The resulting recombinant virus is then used to infect indicator cells containing a *lacZ* gene under the control of the HIV-1 LTR. For testing of RT inhibitors, virus produced in the absence of drug is added to cells pretreated with serial dilutions of drug. β-Galactosidase in infected cells is quantitated using a CPRG-based colorimetric assay. This assay is no longer available for routine clinical use.

In the PhenoSense® phenotypic assay developed by Monogram Biosciences Inc., plasma-derived PR/RT sequences are amplified as one amplicon and inserted into a luciferase reporter resistance test vector (RTV) using restriction enzyme digestion and DNA ligation [28] (Fig. 83.2). Viral stocks are prepared by co-transfecting HEK293 cells with the test vector DNA and an expression vector that produces the amphotropic murine leukemia virus (aMLV) envelope protein. For the testing of protease inhibitor susceptibility, transfected producer cells are incubated in the presence of serial dilutions of drug. Pseudotyped viruses harvested from the transfected cells are then used to infect fresh HEK293 cells. For the assessment of RT inhibitors, virus produced in the absence of drug is added to cells pretreated with serial dilutions of drug. The production of luciferase is dependent on the completion of a single round of replication (infection, reverse transcription, and integration). Drugs that inhibit viral replication reduce luciferase activity in a dose-dependent manner, allowing the quantitative measurement of antiretroviral drug susceptibility (Fig. 83.3). The assay was subsequently adapted to allow the measurement of HIV integrase (IN) inhibitor susceptibility (PhenoSense® Integrase) [29, 30] and, more recently, the measurement of HIV PR/RT/IN inhibitor susceptibility, in conjunction with genotypic resistance analysis, from a single RTV (PhenoSense® GT plus Integrase) [31]. The assay was also adapted to allow assessment of maturation inhibitor susceptibility (Gag assay) for research and drug development purposes [32]. The distinguishing features of various HIV drug susceptibility assays are summarized in Table 83.1.

The recombinant virus assays described above share some drawbacks. Clinically relevant thresholds that define resistance are not known for all drugs (see below). The presence of a minority species of resistant virus(es) may be missed if their relative proportion and/or fitness is below that required for the IC_{50} to shift above the cutoff; the proportion required varies for each drug and mutation pattern. However, both of these limitations (interpretation and detection of minor species) also apply to standard population genotyping assays. Alternative approaches such as traditional clonal phenotypic or genotypic analysis are too expensive and cumbersome for routine clinical use. However, recent advances in "single genome sequencing" methodologies could allow the cost-effective genotypic analysis of minor species if deemed clinically relevant. Partly to minimize the potential for missing the presence of resistant virus, current recommendations emphasize the need to draw a blood sample while an individual is still taking a failing drug regimen to avoid the possibility of archived drug-sensitive virus from outgrowing the resistant variants [33].

Studies that have compared results from different HIV-1 phenotyping assays are limited. Qari et al. tested a panel of 38 samples, many of which were sensitive to all antiretrovirals, in the PhenoSense and Antivirogram assays [34]. Over 90 % of individual results were considered concordant, using a dichotomous scoring system based on susceptibility cutoffs in use at the time of the study. The majority of discordant results had a fold change in IC_{50} values close to the cutoff used. Miller et al. used a panel of 28 specimens, which included a greater proportion with drug resistance, and compared all three assays that were commercially available at that time [35]. Again, the results generally had a good concordance. The most comprehensive analysis comparing PhenoSense and Antivirogram was published by Zhang et al. and demonstrated an improved precision for PhenoSense with nucleoside RT inhibitors [36].

3.3.1 Phenotype Test Interpretation

The interpretation of phenotypic susceptibility assay results is enhanced by relevant thresholds, or "cutoffs", that are

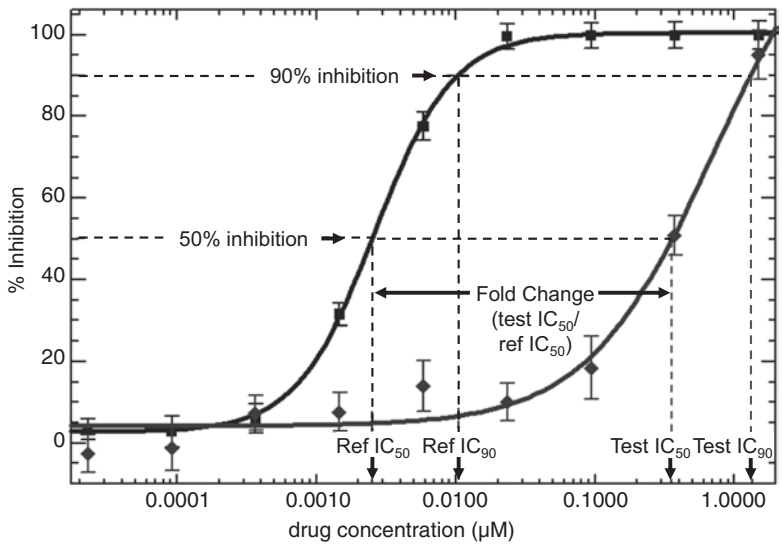

Fig. 83.3 Inhibitor susceptibility curve (PhenoSense HIV assay). Derivation of reference and test sample IC_{50}, IC_{90}, and IC_{50} fold-change values

Table 83.1 Phenotypic assays for HIV protease, reverse transcriptase, and integrase inhibitor susceptibility testing

	ACTG/DOD PBMC [10]	Antivirogram[a] [26]	Phenoscript[b] [27]	PhenoSense [28]	PhenoSense Integrase [29]	PhenoSense GT Plus Integrase [31]
Supplier	Various academic labs	Virco, Belgium	VIRalliance, France	Monogram Biosciences Inc., USA	Monogram Biosciences Inc., USA	Monogram Biosciences Inc., USA
Region of virus tested	All	PR 1–99, RT 1–400	PR 1–99, RT 1–503	PR 1–99, RT 1–305	IN 1–288	PR 1–99, RT 1–400, IN 1–288
		Gag variable	Gag variable	Gag 418–500	RNaseH	RNaseH
Readout	p24 antigen	MTT/cell viability (colorimetric)	β-Galactosidase (colorimetric)	Luciferase (luminescent)	Luciferase (luminescent)	Luciferase (luminescent)
Cells	Donor PBMCs	MT-4	P4 HeLa	HEK 293	HEK 293	HEK 293
Replication competency	Replication competent	Replication competent	Replication defective, single cycle	Replication defective, single cycle	Replication defective, single cycle	Replication defective, single cycle
Recombinant virus construction methodology	N/A	Homologous recombination	Homologous recombination	DNA ligation	DNA ligation	DNA ligation
Amplification sensitivity	N/A	>1000 copies/mL	>1000 copies/mL	>500 copies/mL	>500 copies/mL	>500 copies/mL
Envelope	HIV env from virus tested	HIV (HXB2) env	VSV-G	aMLV	aMLV	aMLV
Turnaround time (weeks)	4–6	3–4	2–3	2	2	2–2.5
Validated according to CLIA/local guidelines	No	Yes	Yes	Yes	Yes	Yes

[a]Discontinued
[b]No longer available for routine clinical use

intended to define the point above which the utility of a given drug begins to decline. "Clinical cutoffs" based on virologic response data from clinical trials provide the most clinically relevant threshold but are also the most difficult to define. To date, clinical cutoffs included in the PhenoSense, PhenoSense GT, PhenoSense Integrase, and PhenoSense GT Plus Integrase HIV assays have been defined for 14 drugs [31, 37–47]. The Phenoscript assay included clinical cutoffs for nine drugs [48, 49] and Antivirogram for four drugs [37, 40, 50–52] (Table 83.2). In the absence of clinical cutoffs, two alternative types of cutoffs have been used. The "assay" cutoff is defined by the intrinsic variability and technical limits of the assay during repeated testing of clinical samples. The "biological" cutoff is defined by an upper limit of the distribution of susceptibility exhibited by wild-type viruses, for example, the mean fold-change +2 standard deviations [53] or the 99th percentile [54]. The clinical relevance of biological cutoffs is limited, however, since the FC value that may be associated with declining virological responses can vary according to the drug. Importantly, the biological cutoff reflects both natural variation in viral susceptibility and inherent assay variability. Thus, such cutoffs may differ among assays that have different intrinsic variability.

3.3.2 Adaptation of Recombinant Virus Assays to Entry Inhibitors

HIV entry inhibitors include peptide inhibitors of virus-cell fusion and small molecules or antibodies that can target the viral envelope protein (Env) or cell-surface proteins (e.g., CD4, CCR5, or CXCR4) to prevent infection of cells [62, 63]. Enfuvirtide (ENF) is a synthetic peptide fusion inhibitor based upon the heptad repeat 2 (HR2) domain in the gp41 subunit of HIV-1 Env. ENF binds specifically to the HR1 domain in gp41 and resistance maps to this region [64–66]. To monitor the emergence of ENF resistance, two of the rapid phenotypic assays (Phenoscript and PhenoSense) that were originally developed for evaluating PR/RT resistance were modified [61, 67]. For Phenoscript, a fragment of the envelope gene (*env*) spanning gp120 and part of gp41 is amplified and co-transfected with an *env*-deleted proviral vector. Recombinant virus is used to infect cells containing an HIV LTR-β-gal reporter gene and expressing CD4 and one or both of the HIV coreceptors, CCR5 or CXCR4. In the PhenoSense Entry assay, the entire *env* gene (gp160) is transferred to an expression vector and co-transfected with a luciferase reporter viral vector. Resulting viral pseudotypes are used to infect cells expressing CD4 and CCR5 and/or

Table 83.2 Phenotypic susceptibility cutoffs

		PhenoSense			Phenoscript[a]			Antivirogram[b]		
Drug class	Drug	Cutoff (FC)	Type[c]	Ref.	Cutoff (FC)	Type	Ref.	Cutoff (FC)	Type	Ref.
NRTI	Abacavir	4.5	C	[39]	8	C	[49]	3.2	C	[52]
	Didanosine	1.3	C	[43]	2.5	C	[49]	2.3	B	[55]
	Lamivudine	3.5	C	[41]	5.5	B	[48]	2.1	B	[55]
	Emtricitabine	3.5	D	[56]				3.1	B	[50]
	Stavudine	1.7	A	[57]	3	C	[49]	2.2	B	[50]
	Tenofovir	1.4	C	[58]	4	C	[48]	2.2	B	[50]
	Zidovudine	1.9	B	[59]	4.5	B	[48]	2.5	B	[50]
NNRTI	Delavirdine	6.2	B	[59]	10	B	[48]	7.7	B	[55]
	Efavirenz	3	B	[59]	5	C	[49]	3.3	B	[50]
	Etravirine	2.9	C	[31]				3.2	B	[50]
	Rilpivirine	2.5	B	[31]				3.7	B	[60]
	Nevirapine	4.5	B	[59]	6.5	B	[48]	6	B	[50]
PI	Atazanavir	2.2	C	[46]				2.1	B	[50]
	Atazanavir/r	5.2	C	[46]	7	C	[49]			
	Amprenavir[a]	2	B	[59]	2.5	A	[49]	2.2	B	[50]
	Amprenavir/r[a]	4	C	[45]						
	Darunavir/r	10	C	[47]				10	C	[51]
	Fosamprenavir/r	4	C	[31]						
	Ritonavir	2.5	B	[31]						
	Indinavir	2.1	B	[59]	2.5	A	[49]	2.3	B	[50]
	Indinavir/r	10	C	[42]	20	C	[49]			
	Lopinavir/r	9	C	[40, 45]	10	C	[48]	10	C	[40]
	Nelfinavir	3.6	B	[59]	3	B	[48]	2.2	B	[50]
	Saquinavir	1.7	B	[59]	2.5	A	[49]	1.8	B	[50]
	Saquinavir/r	2.3	C	[45]	11	C	[49]			
	Tipranavir/r	2	C	[45]				3	C	[37]
INI	Dolutegravir	4	C	[31]						
	Elvitegravir	3.5	B	[31]						
	Raltegravir	2.2	B	[31]						
EI	Enfuvirtide	6.5	B	[61]						

FC: fold change from reference
[a]No longer available for routine clinical use
[b]Discontinued
[c]A, assay/reproducibility cutoff; B, biological cutoff; C, lower clinical cutoff; D, clinical cutoff derived by analogy to critical parameters of lamivudine

CXCR4. Both assays use inhibition of the reporter gene activity to generate IC_{50} or IC_{90} data. Studies using these assays, as well as others, revealed that natural variation in ENF susceptibility can be quite extensive [61, 67]. A clinical interpretation of these differences has been hindered by the lack of studies allowing for the derivation of a clinical cutoff for ENF; therefore, a biological cutoff is used to define a virus as having reduced susceptibility.

Recombinant virus entry assays can also be used to assess resistance to entry inhibitors that target Env interactions with CD4, CXCR4, or CCR5, including attachment inhibitors and chemokine receptor antagonists. For some inhibitors, including the CD4 antibody ibalizumab and the CCR5 antagonist maraviroc (MVC), resistance in a phenotypic assay can be observed as increases in IC_{50} and IC_{90} values and/or as a reduction in the maximum percent inhibition (MPI) obtained, visualized as a "plateau" at which infection can no longer be inhibited further with increasing drug concentrations [68–70].

3.4 Assays for HIV Fitness and Replication Capacity

Viral fitness is defined as the ability of a virus to reproduce within a defined environment. Mutations that confer drug resistance often reduce viral fitness in the absence of drug by interfering with one or more critical steps in the replication cycle. Replication capacity (RC) refers to the ability of a virus to replicate in the absence of drug as compared to that of a wild-type, drug-sensitive control virus. Several methodologies for determining viral fitness have been described, including replication-competent virus growth kinetic assays that

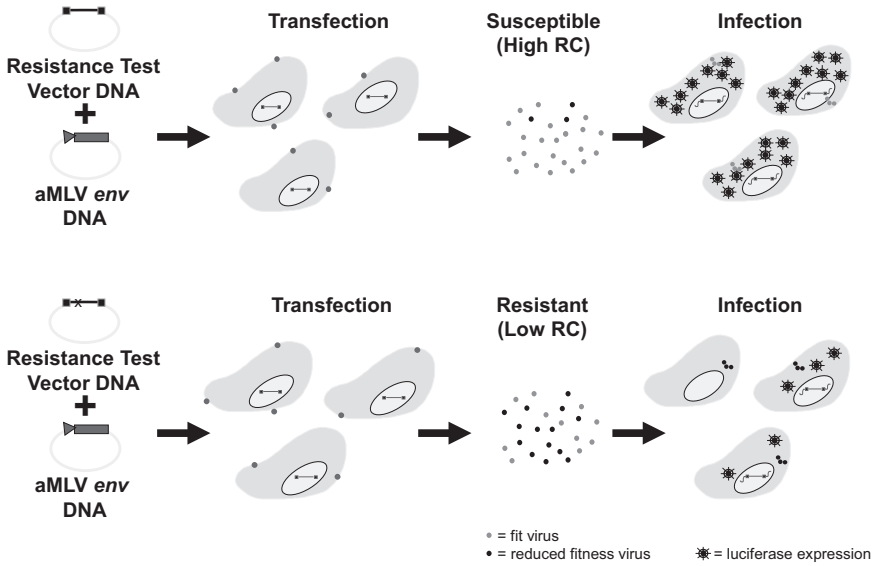

Fig. 83.4 Replication capacity assay (PhenoSense HIV). Drug-resistant viruses often exhibit reduced replication capacity (RC) compared to drug-susceptible viruses

compare the efficacy of viral replication of two or more variants in parallel or competitive cultures. Competitive culture assays measure the proportions of competing viruses over time using a variety of techniques including a recombinant marker virus assay [71] and a heteroduplex tracking assay [72]. A competition assay is regarded by many as the standard methodology to evaluate viral fitness because of its ability to measure the replicative abilities of two viral strains under identical conditions. However, the laborious nature and extended turnaround time of these assays make them impractical for routine clinical use. More rapid, single-cycle, phenotypic susceptibility assays have been adapted to measure RC (Fig. 83.4). In this case, the reported RC only relates to the portion of the amplified sequence transferred to the recombinant virus (i.e., PR and the partial gag and RT sequences included in the amplified fragment), and so the data must be interpreted carefully. Nonetheless there is evidence that if fitness differences are related to changes in PR/RT, the recombinant virus RC assay is a good surrogate of in vivo fitness [73].

Studies have shown that there is a wide distribution of RCs among wild-type HIV lacking phenotypic or genotypic resistance [54, 74, 75]. In general, drug-resistant HIV has been found to possess reduced RC and in vivo fitness, as demonstrated by the reappearance of less resistant virus in individuals whose antiretroviral therapy is interrupted, concomitant with an increase in viral load and decrease in CD4 cell count [73]. However, transmitted multidrug-resistant forms of HIV remain resistant for long periods of time even in the absence of drug pressure and with low viral fitness [75–77], presumably because the reversion rate is slower than that for outgrowth of archived drug-sensitive strains or due to unfavorable (unfit) intermediate forms on the pathway back to a drug-sensitive progenitor [78]. The availability of a convenient RC assay and accumulation of large amounts of data has enabled studies correlating the presence of specific resistance-associated mutations with low RC [79–86]. Such analyses may facilitate the formulation of treatment strategies designed to force the development of certain mutations which also reduce viral fitness [87, 88]. While the clinical utility of measurements of viral fitness or RC for a given individual is unclear, some reports have indicated a correlation between low RC and preservation of CD4 cell counts [74, 75, 89, 90].

3.5 Determining Coreceptor Tropism for HIV-1

HIV-1 infection requires interactions between the viral Env surface glycoprotein (gp120), the cellular receptor (CD4), and a coreceptor (e.g., CCR5 and/or CXCR4) [91]. CCR5 is expressed on primary T-cells and macrophages and is predominately used as a coreceptor by HIV transmitted between individuals and viruses present during early infection [92]. CXCR4 is expressed on many cell types, including primary T-cells, macrophages, thymocytes, and T-cell lines. CXCR4-using viruses are more commonly found in individuals with advanced disease [92]. However, it is not clear whether CXCR4 use precedes and causes more rapid disease progression or is merely the consequence of a change in target cell availability.

The discovery of HIV coreceptors enabled the development of HIV-1 entry inhibitors that target CCR5 in particular, including MVC (Pfizer, approved), vicriviroc (Schering-Plough, development halted), aplaviroc (GlaxoSmithKline, development halted), cenicriviroc (Takeda Pharmaceutical and Tobira Therapeutics, development for HIV on hold), and PRO 140 (CytoDyn Inc.) [62, 63]. The clinical development

of coreceptor inhibitors, and subsequent approval of MVC, necessitated the development of validated assays to determine coreceptor tropism [93, 94]. More recently, gene therapy-based approaches targeting CCR5 have further heightened interest in coreceptor usage and assays to measure it [95].

3.5.1 MT-2 Assays

CXCR4-using viruses can induce the formation of syncytia (syncytium-inducing (SI) virus) when cultured on the CXCR4-bearing MT-2 cell line. MT-2 cells lack CCR5 and are unable to be infected by CCR5-using HIV-1. Thus prior to the identification of coreceptors, CCR5-using HIV-1 isolates were classified as non-syncytium inducing (NSI). Two standardized MT-2 assay approaches have been described to evaluate coreceptor tropism. In one [96], there is a requirement to generate viral stocks from PBMC co-cultures, as described above. These stocks are titrated and can then be used to infect MT-2 cells. Since MT-2 cells express CXCR4 but not CCR5 [97], only SI (CXCR4-tropic) HIV-1 will be able to infect and induce the formation of syncytia. The assays are typically read 14 days or more after infection. Assessment requires microscopic inspection of individual cultures to determine the presence (SI) or absence (NSI) of syncytia. The second method utilizes direct cocultivation of MT-2 cells with an HIV-infected individual with PBMCs, followed by microscopic examination [98]. Prior to the identification of coreceptors, MT-2 assays were a common method of determining HIV phenotype in clinical research settings. Early studies utilizing an MT-2 assay established the SI phenotype as an important marker of disease progression [99]. Despite these findings, the MT-2 assay has not become a routine clinical monitoring test, owing to the time- and labor-dependent nature of the assay process, the lack of ability to directly alter this phenotype by previously available antiretrovirals, the potential drawback that the virus tested is derived from stimulated lymphocytes and not plasma virus and thus may not be representative of circulating virus, the nonquantitative nature of the assay readout (SI or NSI), the variable ability of CXCR4-tropic viruses to induce syncytia, and the potential for some non-CXCR4-tropic viruses to induce syncytia via an alternative coreceptor(s) [100].

3.5.2 Recombinant Viral Assays for Tropism

Entry susceptibility assays (see above) have been modified to enable the determination of HIV coreceptor tropism [93, 94, 101]. Recombinant viruses are used to infect mammalian cell lines expressing CD4 and either CXCR4 or CCR5. One such high-throughput assay (Trofile®, Monogram Biosciences Inc.) [93, 94] has been utilized in the clinical development of coreceptor inhibitors and is commercially available for selecting individuals suitable for MVC treatment. This single-cycle assay utilizes luciferase reporter pseudotype viruses and quantitates luciferase activity as relative light units (RLUs) to assess infection of U87 cells expressing CD4 and either CXCR4 or CCR5. As a confirmatory step, luciferase production must be inhibitable by an antagonist specific for the coreceptor being evaluated. This step is particularly relevant when infection levels are low and result in luciferase activity close to background levels. In June 2008, the original Trofile assay was superseded by an assay with enhanced sensitivity for the detection of minority variants [94]. This improved sensitivity allowed for the earlier detection of emergent CXCR4-using subpopulations in longitudinal samples and further optimized the selection of individuals for CCR5 antagonist therapy [94, 102–105]. The enhanced sensitivity Trofile assay is considered the current benchmark for coreceptor tropism evaluation. A version of this assay that utilizes cell-associated HIV-1 DNA as a template (Trofile® DNA), rather than plasma virus RNA, became available in 2010 to support treatment decisions in the context of virologic suppression [106].

The Tropism Recombinant Test (TRT; VIRalliance) is similar to the original Trofile assay except that a smaller region of the *env* gene (V1–V3) is amplified, and the readout is based on colorimetric assessment of β-galactosidase activity [101]. This assay was to be made available through Eurofins, but is not currently offered for routine clinical testing. The two recombinant tropism assays (TRT and the original Trofile assay) gave largely concordant tropism results (85 %) in a comparative study, with a few unresolved discordances and no evidence of differences in sensitivity [107]. While the V3 loop in the gp120 domain of Env is the major determinant of coreceptor use, regions outside of V3, and even outside of gp120, can also influence coreceptor tropism and thus may account for some discordant results between V3-based assays and those that utilize the entire Env [108].

A number of other recombinant virus-based tropism tests have been developed for research applications or exploratory clinical applications. These include:

(a) The Toulouse tropism test (TTT) which evaluates gp120 and the ectodomain of gp41 cloned from plasma virus or cell-associated DNA [109]. From a comparative analysis of tropism results for 24 samples, 92 % concordance to the enhanced sensitivity Trofile assay was obtained [109].
(b) A promoter-PCR (pPCR) assay in which overlapping PCR is used to assemble a CMV promoter to a population of full-length *env* genes which are then directly co-transfected with an Env-defective luciferase reporter HIV construct to generate pseudovirions, avoiding cloning/recombination steps [110]. Using this assay, results for 9/9 samples were concordant with the original Trofile assay [110].
(c) The VERITROP™ cell-to-cell fusion assay which utilizes a yeast-based homologous recombination approach to clone *env* genes into a HIV vector [111]. A comparative study to the original Trofile assay demonstrated 74 % (56/76) concordant results [111].

3.5.3 Comparison of MT-2 and Recombinant Virus Coreceptor Tropism Assays

There are important differences between MT-2 and recombinant virus assays. These assays typically evaluate HIV from distinct compartments: stimulated lymphocytes versus plasma. MT-2 assays utilize intact virus and recombinant assays evaluate the viral *env* gene. MT-2 assays permit multiple cycles of replication (and possible amplification of viral subpopulations and/or viral adaptation to culture conditions), while recombinant assays limit replication to a single cycle.

An SI result in an MT-2 assay is an established surrogate for HIV-1 CXCR4 utilization. This is supported by limited data examining the relationship between phenotypes determined by the MT-2 assay and the Trofile coreceptor tropism assay. In one study, 11 individuals with HIV determined to be SI in the MT-2 assay [112] had coreceptor typing performed retrospectively with the Trofile assay; virus from all 11 individuals was X4 or dual/mixed (DM (dual: CCR5 plus CXCR4. Mixed: populations of viruses with mixed tropisms that include CCR5- and CXCR4-using viruses)). Luciferase activity obtained on CXCR4-expressing cells infected with pseudovirions from these 11 samples was not uniform but rather varied over a very broad range of RLUs. Further studies will be required to determine whether this is clinically meaningful.

In a second study, the Trofile assay was utilized to determine the coreceptor tropism of virus from individuals prior to entry into a clinical trial of vicriviroc for the AIDS Clinical Trials Group 5211 study [113]. MT-2 assays were performed retrospectively among baseline isolates and revealed only limited discordance between the two assays [114]. Notably, the virus recovery rate among lymphocyte samples processed for the MT-2 assay was low (50 %) compared to the proportion of samples successfully phenotyped by the Trofile assay (>90 %). In a third study, the original and enhanced sensitivity Trofile assays were used to retrospectively evaluate sequential samples from individuals previously evaluated in an MT2 assay. Results were highly concordant and the evolution of coreceptor tropism from R5/NSI to DM/SI over time was noted in both assays [105].

4 Phenotypic Drug Susceptibility Assays for Hepatitis B Virus

Several specific antiviral drugs are now available for chronic HBV infection, including pyrimidine analogues (telbivudine, lamivudine) and purine analogues (tenofovir, entecavir, adefovir). As is the case for HIV, the use of these drugs can lead to the emergence of drug-resistant strains, associated with mutations within the polymerase gene [115] (see also chapter by Stephen Locarnini). With prolonged therapy and continued viral replication, mutations can accumulate and lead to significant cross-resistance between some polymerase inhibitors. Thus it may be important to detect and measure HBV drug resistance to manage the therapy of treatment-experienced HBV-infected individuals. To date, no detectable resistance has been observed following up to 7 years of treatment with tenofovir [116, 117]. However, preexisting adefovir resistance can decrease tenofovir activity [118].

While some HBV cell culture models have been described [119, 120], HBV presents unique challenges due to the fact that no routine robust cell culture system has been established to support the replication of HBV isolates (e.g., for viral spread assays). Therefore, phenotypic assays for the measurement of HBV antiviral drug susceptibility typically rely on several alternative methodologies and are limited to research/clinical research applications.

Phenotyping assays using full-length genomes from parental or mutant laboratory strains have been applied to study HBV resistance in transient assays [121, 122]. Cells able to support transient HBV replication (e.g., HepG2 or Huh7) are transfected with HBV plasmid vector constructs. Intracellular genome replication, dependent on the activity of the parental or altered HBV polymerase, is then compared in the presence and absence of the antiviral drug. Replication is traditionally monitored by Southern blotting; however this technique has limited clinical application due to the cumbersome nature of the readout. Additional concerns include questionable relevance of the behavior of individual mutations in a laboratory virus strain background.

Baculovirus vector-based HBV phenotyping assays to evaluate drug susceptibility have also been described [123, 124]. These approaches allow for efficient transduction of recombinant HBV baculoviruses into hepatoma cell lines. Most HBV drug-resistant variants have been found to replicate in such a system and to demonstrate the expected drug resistance phenotype. However, the procedure is still too cumbersome for routine use in the clinic.

A HBV phenotyping approach that employs PCR amplification of full-length HBV genomes from clinical samples may provide more relevant drug susceptibility information [125]. Clones or quasispecies populations of these genomes can be used instead of parental or mutant laboratory strains in transient transfection studies, using Southern blotting or real-time quantitative PCR approaches to monitor replication [126–129]. A modified version of one assay was commercialized by VIRalliance, but is no longer offered routinely [127]. A variant assay allows the phenotypic assessment of HBV polymerase/RT sequences from clinical specimens of genotypes A to H in the context of a recombinant genotype A HBV backbone [130, 131]. Polymerase/RT sequences are more easily amplified compared to full-length genomes; therefore, this approach facilitates the analysis of clinical samples with lower viral loads.

5 Phenotypic Drug Susceptibility Assays for Hepatitis C Virus

From 2001 through May 2011, HCV infection was treated with a combination of pegylated interferon alpha (peg-IFNα) and ribavirin (RBV) [132]. This entailed a long treatment course with significant side effects that was only approximately 50 % effective for individuals with genotype 1 HCV, the most common HCV genotype in North America [133–135]. Over the past few years, extensive antiviral drug discovery/development efforts have focused on direct-acting antiviral (DAA) agents that primarily target the NS3/4A protease, NS5B polymerase, or NS5A protein of HCV [132]. This has resulted in the approval of a number of different treatment regimens that variably incorporate protease inhibitors (boceprevir, telaprevir, simeprevir, asunaprevir, paritaprevir, grazoprevir), nucleoside (sofosbuvir) or non-nucleoside (dasabuvir) polymerase inhibitors, and NS5A inhibitors (daclatasvir, ledipasvir, ombitasvir, elbasvir, velpatasvir) [132]. Viral strains resistant to most of these compounds can rapidly emerge with suboptimal treatment regimens, given the error-prone nature of the HCV RNA-dependent RNA polymerase and high replication rate of HCV in vivo [136–142]. Thus, as for HIV, DAAs are utilized in combination/coformulated regimens, including with other DAAs with a different mechanism of action and with peg-IFN-α and/or RBV [132].

As for HBV, there is no cell culture system available for the routine culture of clinical isolates of HCV. To date, most in vitro HCV virology studies have been performed using genotype 1 or 2 subgenomic replicons [143–150] or a genotype 2a infectious cDNA clone [151–153]. Adaptive mutations can facilitate replication in cell culture. Replicons with resistance to virtually every compound tested so far can be selected in vitro. Such studies have been highly informative with respect to determination of the location of sites on the protease, polymerase, or NS5A protein that interact with the inhibitor and for the characterization of cross-resistance [139, 154–163]. For example, there appear to be four and possibly five distinct sites where allosteric inhibitors of the NS5B polymerase bind, as determined by the largely non-overlapping sets of mutations selected by the different classes of compound [164]. Variants associated with in vitro resistance to polymerase, NS3/4A protease, and NS5A inhibitors have also been detected in HCV from individuals treated with these inhibitors and largely overlap the in vitro findings [139, 165].

Recombinant replicon systems for assessing the drug susceptibility of plasma-derived HCV have been developed. These assays are currently utilized for research purposes and to support the phenotypic analysis of DAA susceptibility in preclinical and clinical drug development programs [166]. Plasma virus NS3 protease and NS5A or NS5B sequences can be transferred to a luciferase reporter-based replicon vector for susceptibility testing [161, 167–172], such as in the PhenoSense HCV NS3 protease and NS5A and NS5B assays (Monogram Biosciences Inc.; Fig. 83.5). Assay formats are similar to recombinant assays for HIV-1, in that target sequences are amplified from plasma by RT-PCR, transferred to a viral vector, introduced into cells, and cultured with serial dilutions of various inhibitors. Key differences include the requirement for in vitro RNA transcription (since the system relies on RNA, not DNA), typically an electroporation step, rather than transfection, and the use of limited number of cell types (derivatives of Huh-7 cells including those "cured" of HCV infection) which are able to support the high level of replication needed for the transient transfection assay format (Fig. 83.6).

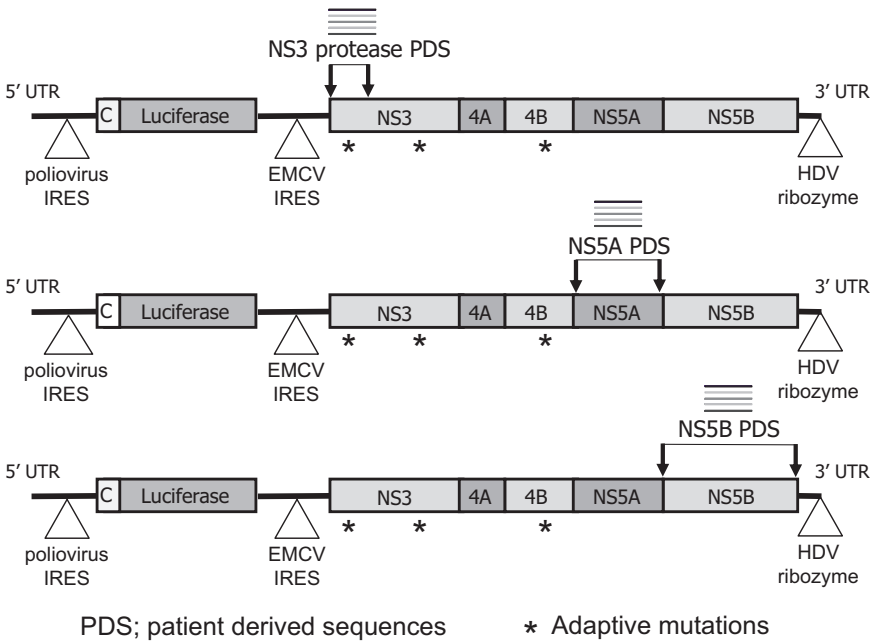

Fig. 83.5 Resistance test vectors for HCV replicon assays (PhenoSense HCV)

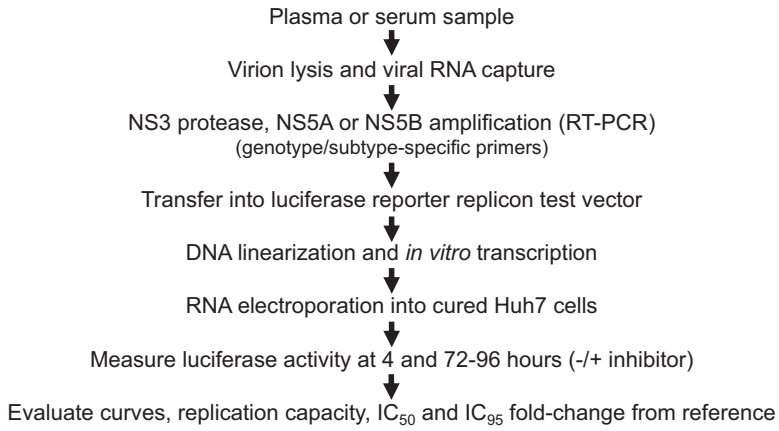

Fig. 83.6 Process flow for HCV replicon assays with clinical samples (PhenoSense HCV)

Challenges for phenotyping HCV clinical samples are related to the extensive diversity between HCV genotypes and subtypes and include (a) the design of primers and RT-PCR conditions that enable the amplification of a high percentage of samples at low viral loads; (b) the relatively low replication capacity of replicons containing some plasma-derived viral sequences, such as NS3 protease regions from protease inhibitor-resistant variants; (c) the lack of replication with some inter-genotypic recombinants, such as non-GT1 NS3 protease regions in a GT1 replicon backbone; and (d) the availability of a limited number of replicon backbones. HCV diversity has also proven challenging for drug development, with a number of inhibitors exhibiting variable potency within and between HCV genotypes. Natural variation in susceptibility to DAAs within a genotype can range from relatively narrow (e.g., within approximately 10-fold for some nucleoside inhibitors) to wide-ranging (e.g., over 1000-fold with some non-nucleoside polymerase inhibitors), in the absence or presence of known resistance-associated variants [167, 173]. However, as high sustained virologic response (SVR) rates can be obtained with combinations of potent antivirals, phenotypic viral resistance assays are not currently appropriate for routine clinical use as they are for HIV-1. Current guidelines do recommend the use of a genotypic viral resistance assay to select appropriate candidates for treatment with simeprevir in combination with peg-IFN-α/RBV or sofosbuvir [174,174b]. Clinical trials have shown that the efficacy of simeprevir/peg-IFN-α/RBV can be substantially reduced when the NS3 protease Q80K polymorphism is detected at baseline in HCV genotype 1a. Similar findings were observed following simeprevir/sofosbuvir treatment of individuals with cirrhosis. In phenotypic assays, Q80K confers an approximate 10-fold reduction in simeprevir susceptibility [175–177]. Guidelines also recommend genotypic viral resistance analysis of NS5A prior to the use of elbasvir/grazoprevir in HCV genotype 1a infected individuals [174b]. The presence of resistance-associated polymorphisms at amino acid positions 28, 30, 31 or 93, that confer at least a 5-fold reduction is elbasvir susceptibility in phenotypic assays, are associated with reduced efficacy in a 12 week treatment regimen. Treatment duration of 16 weeks with RBV intensification is recommended if variants at positions 28, 30, 31 or 93 are identified [174b].

6 Phenotypic Drug Susceptibility Assays for Herpesviruses (HSV, CMV, VZV)

While virus isolation and growth for the clinically important alpha herpesviruses, such as herpes simplex virus (HSV), cytomegalovirus (CMV), and varicella zoster virus (VZV), are technically possible, as with HIV it is wrought with practical obstacles including low reproducibility, long turnaround time, labor intensity, and biosafety concerns. Therefore, traditional plaque reduction assays for HSV [6], CMV [7] and VZV [8, 178] have been adapted for higher throughput [179] or are being replaced by recombinant virus systems [180–182], including some which rely on reporter gene readout such as secreted alkaline phosphatase (SEAP) [183]. Uncertainty about the clinically meaningful level of resistance is a major issue with the use of some of these assays [184, 185], as it is for HIV-1. Plaque reduction assays for the clinical evaluation of HSV-1/2 drug resistance are available from a limited number of reference or specialized laboratories.

7 Phenotypic Drug Susceptibility Assays for Influenza Virus

Phenotypic drug susceptibility assays for intact influenza virus have mainly been limited to plaque assays, often in Madrin-Darby canine or bovine kidney (MDCK or MDBK) cells. These assays have been successfully used to test the amantadine, rimantadine (adamantane derivative M2 ion channel inhibitors), and ribavirin (not approved for influenza

treatment) susceptibility of multiple strains of influenza [186]. Adamantanes are ineffective for the treatment of influenza B viruses, which lack the M2 protein, and widespread adamantine resistance among influenza A viruses has limited their utility this past decade [187].

In the mid-1990s, the advent of potent neuraminidase (NA) inhibitors such as zanamivir and oseltamivir provided new antiviral options for influenza treatment and created renewed interest in assays to assess influenza antiviral susceptibility. Phenotypic assays to measure NA activity were developed and are based on an enzymatic assay of virus particle-associated NA, using fluorescent or chemiluminescent NA substrates [188–191]. Commercial kits (Applied Biosystems), as well as in-house assays, are currently utilized routinely. In these assays, viral stocks are first titrated to select an assay input that is on the linear portion of the enzyme activity curve. An appropriate dilution of virus and drug are then mixed and incubated together, after which the fluorescent or chemiluminescent substrate is added. After incubation, the reaction is terminated and the amount of NA-released product is measured [192]. Fluorescent assays are more cost-effective, while chemiluminescent assays can have shortened incubation times and wider dynamic ranges, but both enzymatic assays are faster and more reliable than plaque assays. Alternative assays using virions pseudotyped with hemagglutinin and/or neuraminidase have also been described and can allow the biosafe evaluation of susceptibility to neuraminidase inhibitors [193–195]. However, for pseudotype as well as the traditional fluorescent or chemiluminescent assays, since some aspects of NA inhibitor resistance are associated with the hemagglutinin protein [196–199], NA enzyme or pseudovirion release assays may not completely reflect the inhibitor susceptibility of the intact native virus. An assay in which HA-expressing cell lines provide HA in trans to pseudotype HA-deleted, green fluorescent protein-expressing influenza viruses may facilitate analysis of influenza antivirals as well as neutralizing antibodies in a reconstituted virus system [199b].

Both fluorescent and chemiluminescent assays are rapid and reproducible and are used clinically as well as for surveillance [200, 201]. Phenotypic testing for neuraminidase inhibitor susceptibility is particularly useful when new viruses arise or new inhibitors become available, such as peramivir. Given the concern about spread of NA inhibitor-resistant influenza viruses, the Neuraminidase Inhibitor Susceptibility Network (NISN) was originally established to monitor resistance around the world using the chemiluminescent assay outlined above. In 2006, the NISN reported that at 3 years post the introduction of NA inhibitors, the detection of resistant viruses was limited (8 out of 2287 samples tested), but required continued surveillance as inhibitor use became more widespread [202]. Indeed, subsequent surveillance efforts by the NISN, the World Health Organization, as well as other groups, using fluorescent or chemiluminescent phenotypic assays, as well as sequence-based assays, identified widespread resistance to oseltamivir in circulating seasonal influenza from late 2007 to early 2008 and in the 2008–2009 season [187]. Viruses that arose late in the 2008–2009 season and that circulated/arose in following seasons through 2013–2014 had a low incidence of resistance on whole (2 % or less globally); however, clusters of resistant viruses identified in a number of communities in different countries warrant ongoing surveillance [187, 203, 204].

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Attributions

PhenoSense and Trofile are registered trademarks of Monogram Biosciences, Laboratory Corporation of America Holdings. Antivirogram is a registered trademark of Janssen. Phenoscript and VERITROP are trademarks of Eurofins VIRalliance Inc., and Diagnostic Hybrids, Inc., respectively.

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