Technology Offer, Institute of Health Carlos III

HIV-BASED RECOMBINANT VIRAL CLONES AND USE THEREOF IN ANALYTICAL METHODS: KNOW HOW

SUMMARY

The technology offered is based on the generation of recombinant viruses based on a full-length HIV-1 clone (NL4.3, Adachi et al 1986) in which some changes have been produced.

MAIN APPLICATIONS

As described in the brochure five applications are possible

a. Phenotypic resistance to antiretroviral drugs.
b. Fitness assessment.
c. Determination of viral tropism.
d. Titration of neutralizing antibodies.
e. Evaluation of new antivirals.

BROCHURE CONTENT

1. General information about the technology.
2. Brief summary and description.
3. Which are the advantages of these constructs.
4. Main applications of the recombinant virus system.
5. Costs and practical aspects.

REQUESTED COLLABORATION

We are seeking licensees for application or interested in a Know How licensing agreement /collaboration agreement for further development of our technology or its extension to other applications.

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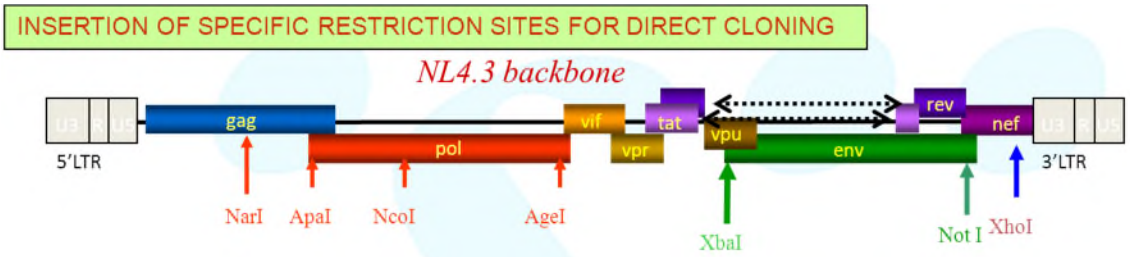
1. GENERAL INFORMATION ABOUT THE THECNOLOGY

HIV-BASED RECOMBINANT VIRAL CLONES AND USE THEREOF IN ANALYTICAL METHODS

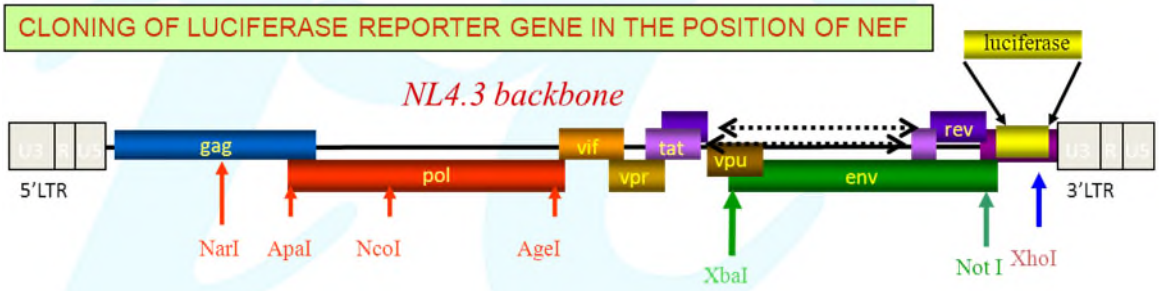
2. BRIEF SUMMARY AND DESCRIPTION

The technology is based on the generation of recombinant viruses based on a full-length HIV-1 clone (NL4.3, Adachi et al 1986) in which the following changes have been produced.

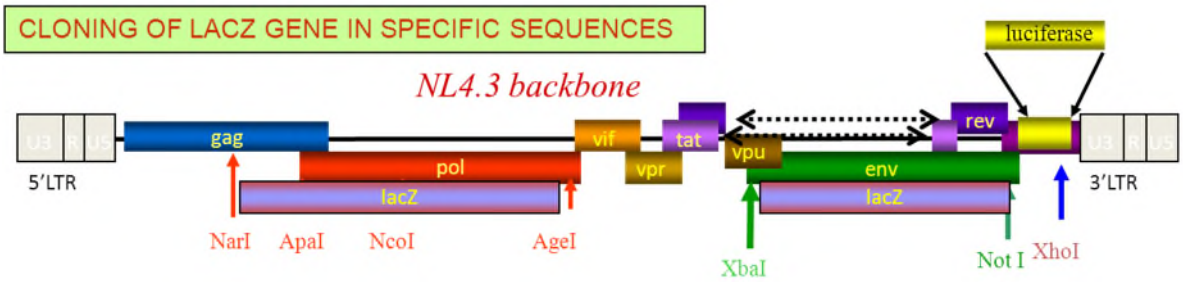
- Introduction of specific ***enzyme-restriction sites*** along the viral vector



- Deletion of the sequences corresponding to the ***nef*** gene and replacement by the renilla-luciferase gene in this position

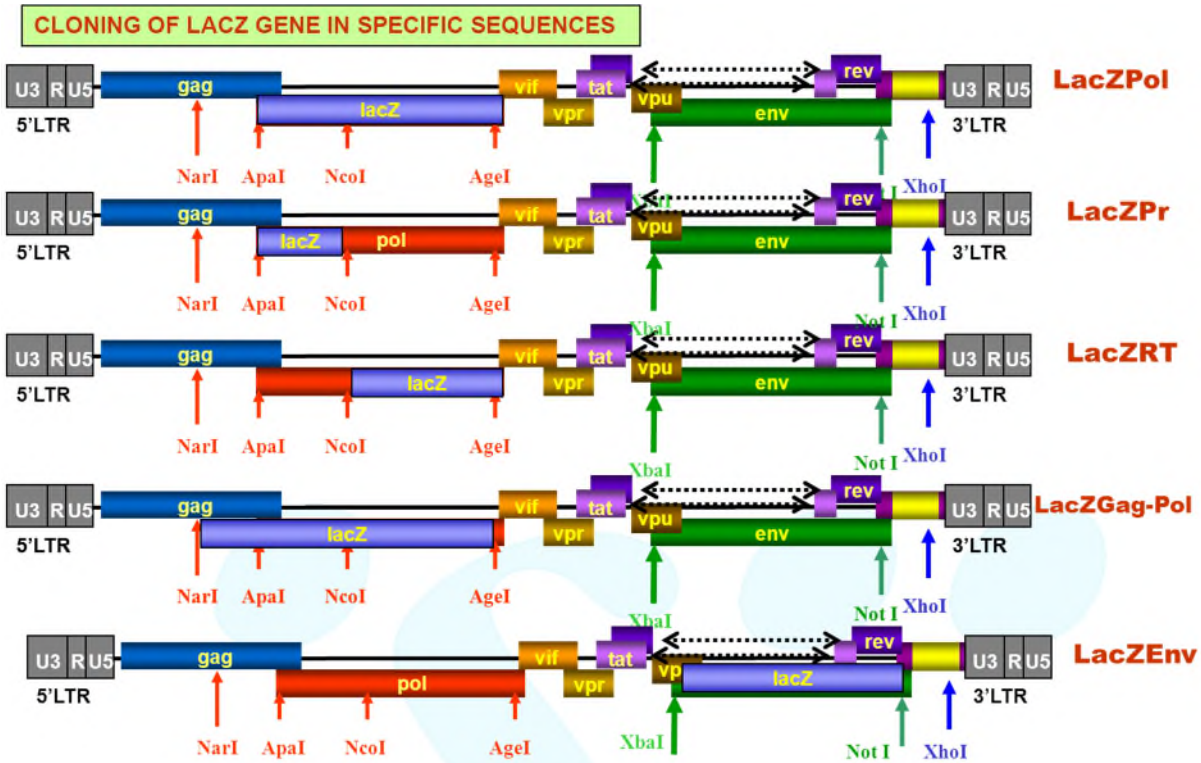


- Replacement of specific viral sequences by the ***Lac-Z gene***



The final goal of these modifications is to generate a collection of viral vectors in which specific genes (gag, pol, env) are deleted (we will call them "target vectors"). All these target vectors carry the renilla-luciferase gene in the position of nef and the Lac-Z gene has been cloned in the position of the deleted HIV-1 fragment. We have generated the following "target vectors".

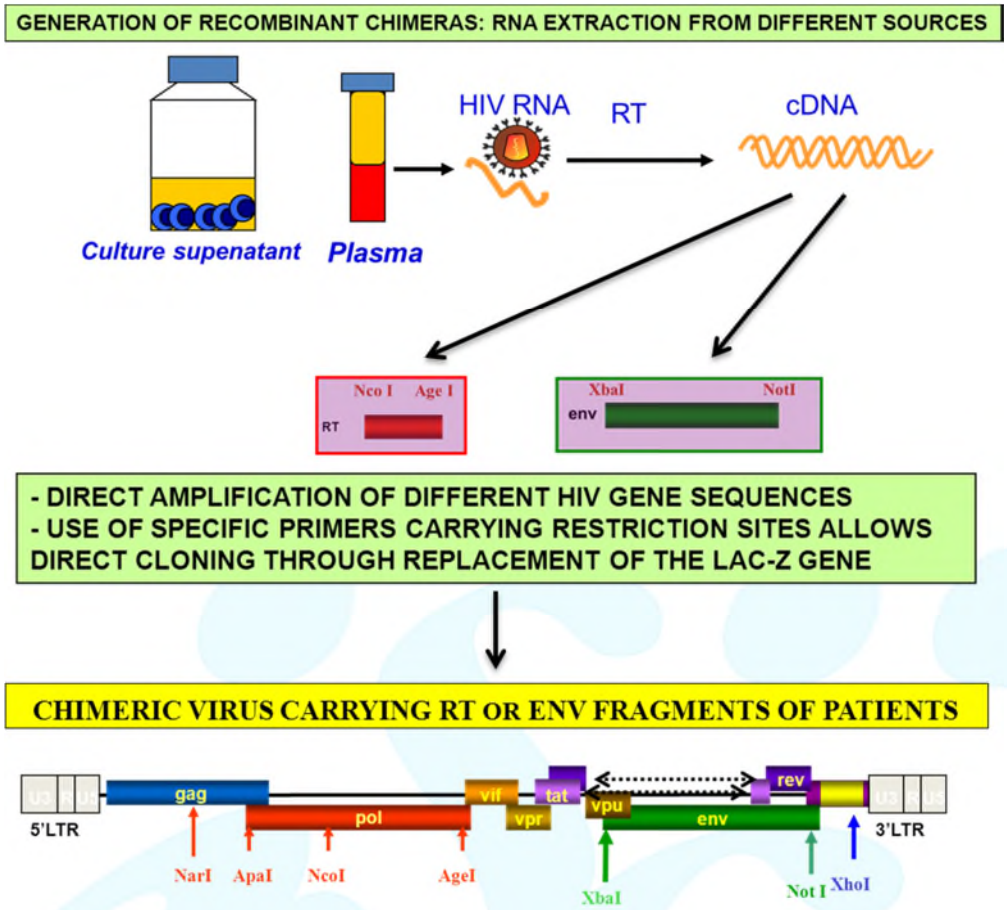
TARGET VECTORS WITH LAC-Z INSERTIONS



3. WHICH ARE THE ADVANTAGES OF THESE CONSTRUCTS?

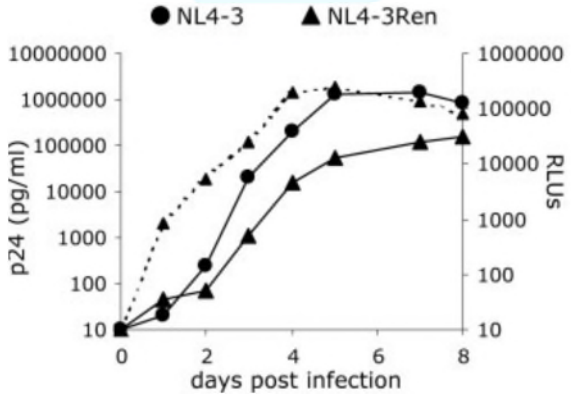
Testing specific patient's viruses

In the different "target" vectors is possible to clone in an easy way similar fragments corresponding to HIV-infected patients. Such sequences are generated by RT-PCR from plasma or other sources (genomic DNA, cell cultures…). Using this methodology 1 ml of plasma with viral load above 1.000 RNA copies is enough to get amplification of this/ these fragment(s). Thus, combining amplification, digestion with restriction enzymes and fragment cloning we generate "chimeric viruses" carrying fragment(s) from one particular HIV-1 patient that confer its (their) phenotypic properties. For example, through cloning of the reverse transcriptase sequence of the patients we can test the profile of resistance to antiretroviral drugs or by replacing the envelope gene we can assess the tropism.



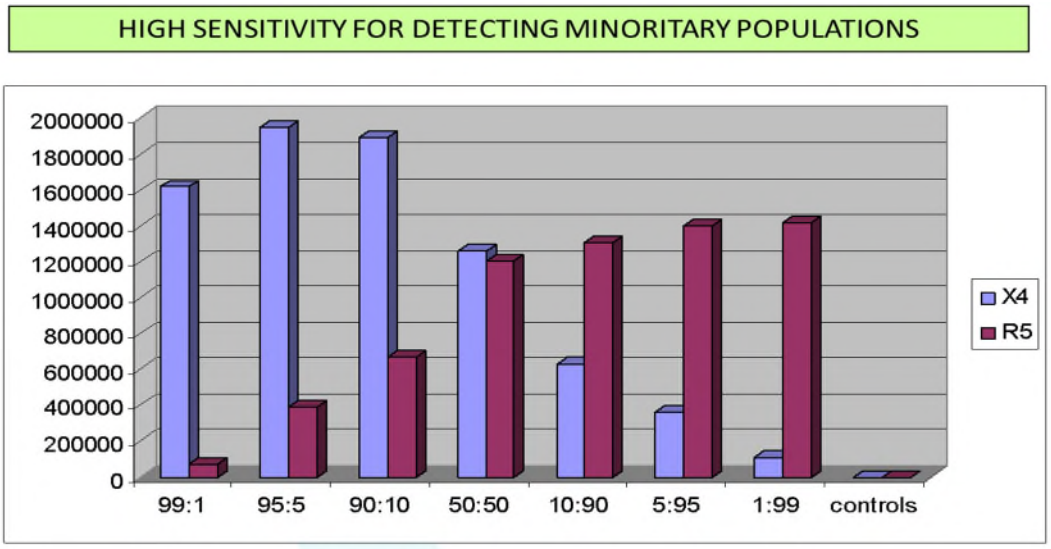
Detection system

The insertion of the renilla luciferase gene in the position of nef gene presents two major advantages: first, luciferase expression allows tight monitoring of the replication, in a very efficient and cheap way. The cost of luciferase detection is 20 times less than assessment of Gag-p24 production and can be assessed 24 hours after infection. Besides, the sensitivity of the technique allows detection of viral replication in a 96 wells plate and the system could be upgraded to 384 wells.



Replication kinetics of wild-type NL4-3 (*) as compared to the reporter virus NL4-3Ren (~). MT2 cells were infected and viral replication measured quantifying HIV-1 p24 antigen (pg/ml) production in the supernatant (solid line). Luciferase activity was also detected in cell lysates from MT2 cells infected with the reporter virus (dashed line).

The second advantage is based on the fact that viruses produced from these constructs are fully competent and can perform several cycles of replication in vitro. This characteristic allows amplification of minority variants. For example, if we have a viral population displaying an X4 tropism we can detect such minor variant after several cycles of infection



Detection of minoritary R5 or X4 quasispecies (1%)
-R5 and X4 clones were infected at different proportions
-Target: U87CD4+CXCR4/CCR5
-Background luciferase level 200 RLU

Use of the Lac-Z gene

Insertion of the Lac-Z gene represents a major advantage by two reasons. First, we avoid contamination with wild type sequences, a common issue when a cloning is performed in a wild type virus because a mix between wild-type and chimeric viruses are generated. In our system, wild type sequences are excluded because they have been replaced by the lac-Z gene. Besides, during the cloning process the proportion of blue bacterial colonies due to persistence of Lac-Z gives us an excellent quality control. If many blue colonies are present then the ligation process has not been efficient. On the contrary, a high proportion of white colonies indicates that cloning of the gene fragment from the patient has been particularly efficient.

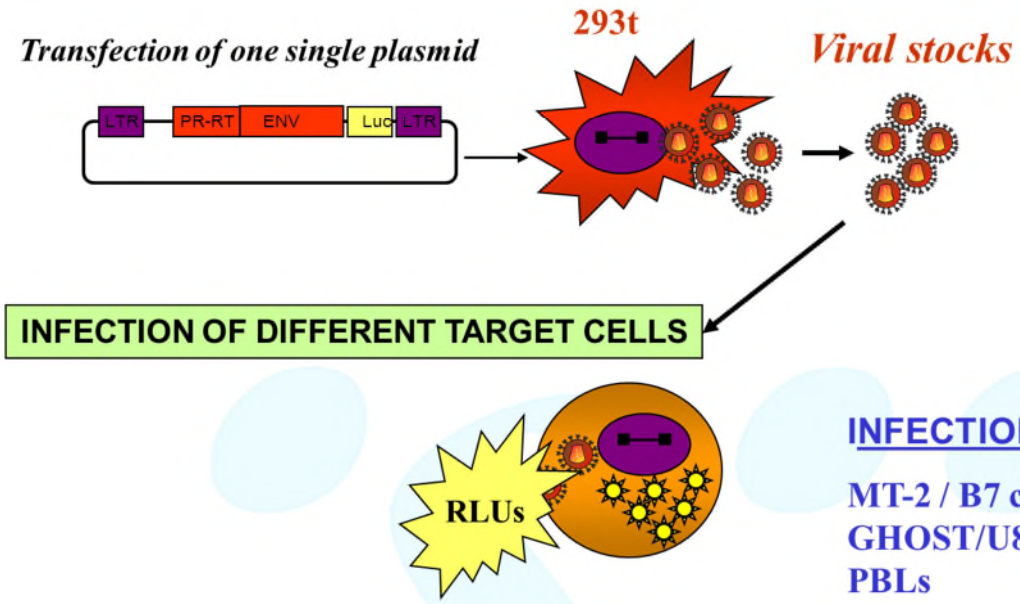
Viral populations and clones

The method has been designed to produce viral populations but working with viral clones is also possible.

Viral production

Finally, the recombinant viruses are generated by transfection of a single plasmid producing cells, usually 293-T that are used as factories for viral generation. Supernatants are collected and used to infect different cell types. Of note, the chimeric viruses generated are able to infect both cell lines and natural cells such as lymphocytes, macrophages or dendritic cells.

PRODUCTION OF VIRAL STOCKS BY TRANSFECTION IN 293T CELLS



MAIN ADVANTAGES OF THE CONSTRUCTS

- Possibility of testing patient specific's viruses.
- Detection system: Quick, cheaper and reliable.
- Better cloning with the use of the Lac-Z gene.
- Both viral populations and clones can be produced.
- Viral production

4. MAIN APPLICATIONS OF THE RECOMBINANT VIRUS SYSTEM

As described in the brochure five applications are possible

f. Phenotypic resistance to antiretroviral drugs
g. Fitness assessment
h. Determination of viral tropism
i. Titration of neutralizing antibodies
j. Evaluation of new antivirals

However, some of these applications are not useful at this moment. A critical assessment of these five applications is described below.

a. Phenotypic resistance to antiretroviral drugs

The system was initially built up to provide a method to assess resistance to antiretroviral drugs in HIV-infected patients. At that time (1999), the number of antiretroviral drugs was limited, cross-resistance among drugs in the same family common and the rate of failure reached 20% of patients/year. When treatment failure against the first combination of antiretroviral drug was produced the choice for a second combination was extremely limited and it was necessary to accurately demonstrate susceptibility of each patient to this new and last chance and try to rescue some of the compounds previously used.
This scenario has changed dramatically. Today, more than 20 different antiretroviral drugs belonging to five different families are in the market and their potency has increased together with a decrease in severe secondary effects. In accordance with these data treatment failure due to resistance emergence has decreased and currently less than 3% of patients develops drug resistance on a yearly basis. Of note, the possibility to replace one failing treatment with a new combination of effective antiretrovirals is now easy.
Finally, genotypic testing has been developed with the generation of large database in which phenotypic susceptibility has been correlated with different patterns of mutations. Accordingly, resistance testing is currently performed with genotypic methods and is mainly performed in naïf patients to rule out the transmission of resistant HIV strains.
In conclusion the use of the Recombinant Virus System for phenotypic testing is not useful anymore in the clinical setting.
On the contrary, at pharmaceutical research level, when a new drug belonging to a given group of antiretrovirals is generated it remains important to check its activity against resistant viruses to other drugs of the same family. In this context we have generated a collection of viral clones carrying different mutations of resistance to non-nucleoside reverse transcriptase inhibitors, nucleoside/nucleotide reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, integrase inhibitors and Maraviroc. Overall, a broad collection of resistant clones has been produced in our laboratory and can be tested against new hits to increase their added value. This panel of resistant viruses remains a major tool that is sometimes requested to analyze cross-resistance of new antiretroviral drugs.

b. Fitness assessment

Our group and others have performed elegant work on the loss of fitness in the context of viral resistance to different antiretroviral drugs. However, this approach has been always considered in an academic research context and provides few relevant information in the clinical setting.

c. Determination of viral tropism

Viral tropism is determined by the use of the CCR5, CXCR4 receptors or both. Accordingly, viral strains are classified as R5, X4 or du-tropic (R5X4) viruses. Development of X4 variants has been associated with a poor prognosis and sharp decline in the number of CD4 lymphocytes. It was therefore interesting –but not essential- to know the tropism of predominant viral strains in a given patient as a prognostic factor.
This picture has changed since the development of CCR5 inhibitors because according to regulatory authority (FDA, EMA) only patients displaying an R5 phenotype should be treated with these drugs. Moreover, the risk of a switch from R5 to X4 tropism has been evoked as a risk when patients carrying a mixture of R5 and X4 viruses are treated with these drugs. In clinical practice, before starting treatment with antagonist of CCR5 a tropism test should be performed in every patient.
Genotypic testing has severe limitations because no specific patterns of mutations of resistance to this family of drugs have been identified. The problem is that in practice the only company performing phenotypic tests of tropism is based in San Francisco (Monogram) and besides the issue of sending infected samples to California, the cost of the test is high (>600$/sample). To overcome this difficulty different algorithms to predict viral tropism have been developed and in practice, many clinicians use genotypic sequence of the V3 loop to predict tropism in their patients.
The predictive value of current algorithms is relatively good for some HIV-1 variants but very poor for viruses belonging to non-B clades. Taking into account that up to 40% of patients carry these viral strains in some regions of Spain phenotypic testing still has a chance to be applied in the clinical setting. If a phenotypic test could be in place at a reasonable price, probably it would be used by many clinicians before treating their patients with CCR5 antagonists. The recombinant viruses of the know how offered can be used to perform that task.

d. Titration of neutralizing antibodies

This approach was originally considered from a basic research perspective to study neutralizing antibodies of potential interest in HIV research. However, the development of new vaccine prototypes aiming at the induction of neutralizing antibodies has increased the utility of this application.
On one hand, the growing field of vaccines triggering humoral responses against HIV requires appropriate testing of neutralizing activity. Paradoxically, these methods were not normalized until recently. Classical tests for titration of neutralizing activity in patient´s sera are expensive, time-consuming and with low reproducibility. The development of different models of recombinant viruses allows accurate evaluation of neutralizing activity against HIV. However, many different systems have been set up by the different laboratories. Between 2004 and 2010 an international consortium led by the FP6 program has allowed a comparison of the different assays to titrate neutralizing antibodies among the different groups. Our laboratory has participated in this validation and our results are highly concordant with the laboratories and methodologies that are considered as the gold standards in the field. In particular, an excellent correlation has been found between our Recombinant Virus System and those of David Montefiori (Duke University, supported by the Gates Foundation), Vicky Polonis (NIH) and the company Monogram.

In the table shown below the degree of concordance is analysed by the number of fold difference for titration of a given antibody against a panel of viruses.

TriMab		Pseudovirus based assay							
		Plasmid						Culture sup.	
	LABORATORY	Montefiori 2	Polonis 5B	Morris 10	Hendrix 6A	Jassoy 1	Monogram 4B	Alcami 12	Monogram 4A
	Montefiori 2	0	3	1	6	2	4	3	2
	Polonis 5B	3	0	2	3	4	1	1	4
	Morris 10	1	2	0	7	5	6	5	5
	Hendrix 6A	6	3	7	0	6	2	3	7
	Jassoy 1	2	4	5	6	0	6	5	2
	Monogram 4B	4	1	6	2	6	0	2	6
	Alcami 12	3	1	5	3	5	2	0	6
	Monogram 4A	2	4	5	7	2	6	6	0
	score	21	18	31	34	30	27	25	32
	virus	10	8	7	11	10	10	9	11
	score/virus	2,10	2,25	4,43	3,09	3,00	2,70	2,78	2,91

The highest degree of global concordance considering all the viruses in the panel is 0, and the absolute lack of concordance is 36. A 10% level of discrepancy (3-5 points) can be considered as very good reproducibility among laboratories. As shown, our group fit an excellent level of agreement with the leaders in the field thus supporting the consistency of our system.

In the last years our group has been a reference laboratory to asses neutralization activity in four vaccine trials developed in Spain. In these tests the sera from patients are incubated with different viral strains and their neutralization capacity assessed. Two parameters are important and should be evaluated: the intensity of the antibody response that is measured as the dilution of sera that neutralized 50% of infection (IC50). The broadness of the response that is assessed as the capacity of a given sera to neutralize a panel of viruses with different origin. Developing broadly neutralizing antibodies is the holy grail of a future vaccine. Thanks to our previous experience and participation in different trials we have developed different panels of HIV strains that cover the spectrum of viruses that should be neutralized by an effective vaccine. Again, these collections of recombinant viruses are a major added value of the work performed along these years.

It is certain that in the following years an increasing number of clinical trials will be performed and this represents an excellent opportunity to apply this technology.

e. Evaluation of new antivirals

Probably this development of the Recombinant Virus System has been the most productive. It was not considered as a major application of the model because we initially focused on the characterization of viral strains of HIV-infected patients from different perspectives (tropism, resistance, fitness…). However, we notice that this system was extremely useful to evaluate and screen for potential antiviral compounds. Actually, more than 5.000 compounds from different origin have been analyzed in the last five year by our group. The system we use has major advantages as compared to other biological or enzymatic tests.

- It represents a biological test in which toxicity of a given compound can be evaluated in parallel to its antiviral activity thus determining Inhibitory Concentration 50 (IC50), Cytotoxicity concentration 50 (CC50) and efficacy index (IC50/CC50) in the same experiment.
- The use of luciferase expression as the outcome of viral replication decreases the cost of these experiments in comparison with classical tests of reverse transcriptase activity or p24 antigen detection by 10-20 fold.

- The sensitivity of luciferase detection allows evaluation in a microplate format (96 well that could be upgraded to 384 well plates) and shorten the duration of the test when compared with classical cytotoxicity assays from 7 days to 24 hours.
- The test adapts to different scenarios and displays: it can assess chemical compounds, plant extracts, nanoparticles, drugs currently used for other purposes and different targets can be employed, including lymphocytes, dendritic cells or macrophages that are the natural targets of HIV infection.
- Again, the broad collection of recombinant viruses generated in the laboratory allows full characterization of the antiviral activity of a given compound

Usually at a first step we measure the IC50, CC50 and efficacy index. If these parameters are interesting we proceed with further steps that are summarized:

- Characterization of the mechanism of action of the compound, and in particular the step of the viral cycle that represents the target of the drug. We have developed different viral constructs and molecular biology approaches through which we can define the target in the virus: direct activity on viral particles, inhibition of entry (through CD4, co-receptor interactions or fusion), retrotranscription, nuclear transport, integration, transcription or post-transcriptional steps.
- Activity against different panels of resistant viral clones, which represent an important added value for a compound belonging to previous families of antiretrovirals.
- Antiviral activity in natural targets such as lymphocytic subsets or macrophages.

MAIN APPLICATIONS

Phenotypic resistance to antiretroviral drugs

A collection of viral clones carrying different mutations of resistance has been generated. This panel of resistant viruses remains a major tool to analyze cross-resistance of new antiretroviral drugs.

Determination of viral tropism

Before starting treatment with antagonist of CCR5 a tropism test should be performed in every patient. The recombinant viruses covered by the know how offered can be used to perform that task at a reasonable price.

Titration of neutralizing antibodies

-Our results are highly concordant with the laboratories and methodologies that are considered as the gold standards in the field.
-Different panels of HIV strains that cover the spectrum of viruses that should be neutralized by an effective vaccine have been developed.

Evaluation of new antivirals

-The system has many advantages as compared to other actual tests (biological test, low costs and time, sensitivity, versatility, …).
-A broad collection of recombinant viruses generated in the laboratory allows full characterization of the antiviral activity of a given compound

5. COSTS AND PRACTICAL ASPECTS

Our group has acquired the technical background to perform previously described tests. We have mainly collaborated in academic studies and clinical trials but occasionally collaborations with biotech and pharmaceutic companies have been perfomed with success.

In our institution we have the facilities requested to perform these tests: BL3 laboratories, different PCR rooms to handle clinical samples, luminometers and molecular biology equipments including sequencing and confocal microscopy platforms.

Trained technical personnel and senior researchers that developed this system work in the lab and have performed continuous improvement of the techniques here described. An extremely valuable collection of recombinant viral clones has been produced along these years and represent and added value for all the described techniques.

The biological material required from patients is usually plasma, thus allowing transport without freezing provide the sample is received in the laboratory in 24 hours after extraction. Processing in PPT tubes is compatible with all the techniques proposed and represent a useful tool for transport of samples at RT. We can also isolate viral sequences from cell DNA.

In the following table it is shown a preliminary approach on the time requested to perform the different techniques and the estimated cost that obviously depends on the number of samples that are processed. We have not included phenotypic resistance testing or fitness assessment because we consider that these developments are not useful at the current time.

Technique	**Sample**	**Time**	**Cost in Euros**	**Observations**
Tropism, phenotype	1 ml plasma	3 weeks	250-300	Genotype is included
Titration of neutralizing activity	2 ml sera	1 week	50	
Antiviral activity. Screening	Compound (amount variable)	1 week	75	Variable depending on the number of compounds.
Antiviral activity Panel of resistant viruses	Compound (amount variable)	2 weeks	300	
Antiviral activity In lymphocytes	Compound (amount variable)	2 weeks	100	
Antiviral activity Mechanism	Compound (amount variable)	4-6 weeks	600-1000	To be discussed in each situation