Trofile™ & Tropism

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THE MARK OF INDIVIDUALIZED THERAPY™

trofile™
CO-RECEPTOR TROPISM ASSAY

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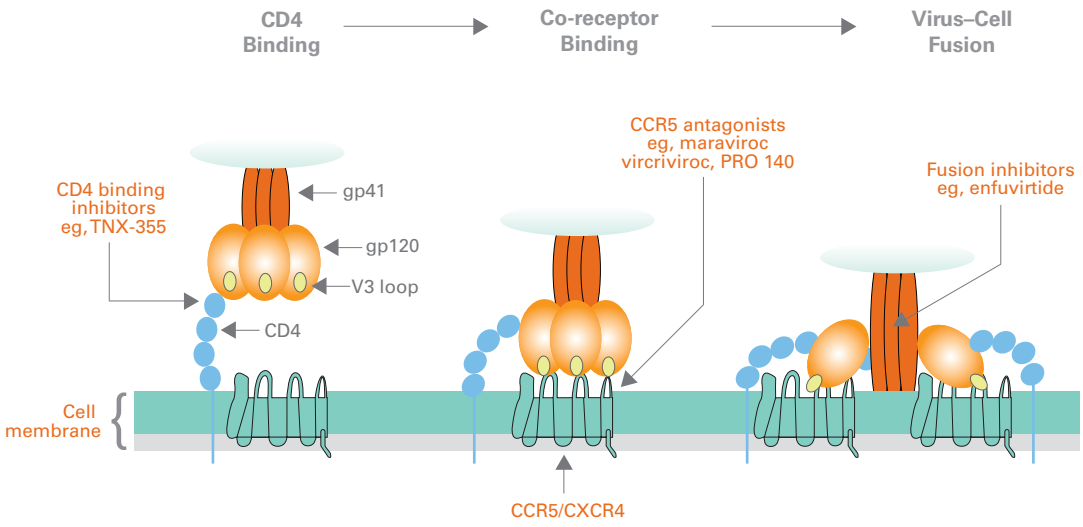
Trofile™ and Tropism

1. Introduction to Trofile and Tropism

1.1 HIV-1 viral entry is mediated by chemokine receptors

For HIV, the first step in its replication cycle is to target and infect its host cell, the CD4+ lymphocyte. It does this using a complex set of interactions with viral proteins displayed on the surface of the viral envelope and receptors on the surface of the CD4+ cell. The viral envelope protein is comprised of two glycoproteins called gp41 and gp120.

Viral entry is proposed to occur in the following manner (Figure 1). The gp120 has an affinity for the CD4+ receptor, which is expressed on the cell surface of the CD4+ cell. The binding of gp120 to the CD4 receptor causes distinct changes in the shape of the gp120 protein. This conformational shift in protein structure reveals a previously hidden binding site for a chemokine co-receptor (either CCR5 or CXCR4) on the target cell surface. Once gp120 binds to the co-receptor, this causes a conformational change in the gp41 protein allowing the insertion of a fusion peptide from the gp41 protein into the host's membrane. This insertion of the fusion peptide draws the virus and CD4+ cell close enough together to allow the virus envelope and the cell membrane to fuse. Once this happens, the viral core can enter the CD4+ cell and the infection begins.



Adapted from Moore JP, Doms RW. *Proc Natl Acad Sci U S A.* 2003;100:10598-10602.

Figure 1. HIV attachment, co-receptor binding, and fusion represent validated targets for inhibition of infection by HIV-1.

1.2 The natural role of chemokine receptors

Chemokines are a collection of polypeptides that have an important role in the inflammatory response where they provoke the migration and activation of phagocytic cells and lymphocytes.[1] Both CCR5 and CXCR4 are chemokine receptors that regulate the cell's immune response through a second messenger system. The specific role of the CCR5 chemokine co-receptor is unclear, as individuals who do not express functional CCR5 co-receptors appear to have normal immune function and have a normal life expectancy.[2] CXCR4 co-receptors in contrast seem essential for development. Genetically modified mice in which the CXCR4 gene is made nonfunctional die in utero.[3]

1.3 Tropism

Co-receptor tropism is defined as the ability of a particular HIV-1 virus to infect a target cell using a specific co-receptor.[4] HIV-1 viruses can be characterized into four broad classifications based on their tropism status.

CCR5-tropic: Viruses or virus populations that can use only the CCR5 chemokine co-receptor to infect $CD4^+$ cells.

CXCR4-tropic: Viruses or virus populations that can use only the CXCR4 chemokine co-receptor to infect $CD4^+$ cells.

Dual (D)-tropic: Viruses or virus populations that can use either the CCR5 or CXCR4 co-receptors to infect $CD4^+$ cells.

Mixed (M)-tropic: Virus populations that may contain various combinations of R5 virus, X4 virus, and/or dual-tropic viruses.

1.4 Clinical relevance of tropism

For reasons not fully understood at present, most cases of primary HIV-1 occur with the R5 virus.[5,6] This is even true if the source patient had evidence of CXCR4-utilizing virus (ie, dual/mixed virus and or X4 virus).[5,6] During the early stage of HIV-1 infection (as determined in clade B HIV-1 infections), the R5 virus predominates. Over 80% of individuals with early stage HIV-1 infection have R5 virus.[7,8] However, as HIV-1 disease progresses, the prevalence of CXCR4-utilizing viruses gradually increases.[9] This shift is typically characterized by the emergence of dual-tropic virus (a virus that can utilize both CCR5 and CXCR4 co-receptors) and not simply the emergence of a pure X4 virus. Pure X4 virus populations are rare and account for only 0.1% of the treatment naive population[7,8] and 3% to 4% of the treatment experienced group.[9-11] About 50% of people with HIV-1 that progresses to AIDS demonstrate only R5 virus throughout the entire course of HIV-1 disease.

When dual and or mixed populations of HIV-1 viruses are detected by Trofile, the assay will report the presence of dual/mixed viruses. The current assay is unable to distinguish between these populations of viruses.

1.4.1 Treatment experienced patients

HIV-1 individuals with stable HAART (highly active antiretroviral therapy) failure exhibit higher levels of dual/mixed virus than treatment naive individuals with the same $CD4^+$ count. It is unclear at present whether the higher levels of CXCR4-utilizing viruses are driven by HAART therapy or reflective of the underlying degree of disease progression (Table 1).

Table 1. The prevalence of both R5 and X4 viruses in treatment naive and treatment experienced HIV-1 infected patients

Study	Population	No. Patients	R5 virus	Dual/mixed virus	X4 virus
Homer cohort[7]	Naive	979	82%	18%	<1%
Chelsea Cohort[8]	Naive	402	81%	18%	<1%
ACTGA5211[9]	Experienced	391	50%	46%	4%
MOTIVATE 1 & 2[10]	Experienced	2,560	56%	41%	3%

1.4.2 Effects of co-receptor antagonists on tropism

The possible effects of co-receptor antagonists on HIV-1 tropism have been the subject of intense study. Three potential outcomes have been discussed: 1) viral suppression, 2) unmasking and emergence of CXCR4-utilizing virus, and 3) tropism switching.

1. The pairing of the appropriate co-receptor antagonist with the appropriate HIV-1 tropic strain will result in a sustained suppression of the virus. This has been demonstrated in clinical trials with maraviroc [BID] plus optimized background therapy in patients with R5 virus (MOTIVATE 1 and 2). These patients had significantly decreased viral loads (–1.95 and –1.97 mean HIV-1 RNA $\log_{10}$) and increased $CD4^+$ cell counts (+102 and +111 cells/mm^3) after 24 weeks of treatment (MOTIVATE 1 and 2 respectively, see Table 2).[11,12]

2. Unmasking is the term used when a minority population of HIV-1 exists below the level of detection for the Trofile assay but is revealed once the major HIV-1 strain is suppressed with co-receptor antagonists. For example, a patient may be designated as having R5 virus, but within this patient's population of HIV-1 viruses is a minority population of CXCR4-utilizing viruses (ie, dual and/or X4 viruses). Once the R5 variant has been suppressed, it is possible that this minority CXCR4-utilizing virus population could expand to become the predominant HIV-1 strain. It has not yet been determined whether the emergence of CXCR4-utilizing virus in the setting of CCR5 antagonist therapy is associated with accelerated disease progression. If this is so then monitoring tropism in the setting of CCR5 antagonist failure may be clinically important.

3. Switching is the term used when a virus population alters tropism. An example is when an R5 virus population develops a CXCR4 tropic component. Switching often occurs during the progression of HIV disease, as evidenced by the increase in dual-tropic viruses in late-stage disease. The question arises that if one variety of co-receptor is blocked by a specific antagonist, might the virus population shift tropism to make use of the co-receptor that is not blocked?

   Data from the MOTIVATE 1 and 2 trials with maraviroc plus optimized background therapy indicated that, at week 24, approximately 7% of the enrolled patients who had R5 virus at enrollment had dual/mixed virus at treatment failure.[11,12] Whether this failure is due to switching or emergence is undetermined at this time.

2. Measurement of Tropism

2.1 Trofile™: A rapid phenotypic assay to measure HIV-1 co-receptor tropism

Trofile was developed by Monogram Biosciences and is CLIA (Clinical Laboratory Improvement Amendments) validated to determine HIV-1 co-receptor tropism in clinical or patient management settings in the United States.[13]

Trofile is a single-cycle recombinant virus assay in which a pseudovirus is generated from full-length envelope (*env*) genes derived from the patient's virus population.

RNA isolated from the patient's HIV-1 is reverse transcribed, and the resulting *env* cDNA amplified by PCR using env-specific primers. These amplified products that represent the diversity of viral *env* sequences are placed into an env-expression vector. The env-vector's role is to generate the Env proteins from the patient's HIV-1.

Secondly, a replication defective HIV-1-genomic vector has been modified so that the *env* region is removed and replaced with a luciferase cassette (indicator cassette). This vector's role is to generate virus particles, but as it cannot generate envelope proteins, the resulting virus particles are not infectious.

Thirdly, to generate the pseudoviruses, both the env-expression vector and the HIV-1-genomic vector are cotransfected into a producer cell (human embryonic kidney cell, HEK293). The HIV-1-genomic vector generates virus particles and uses the patient's Env proteins generated by the env-expression vector to finish the assembly of the pseudovirus.

Finally, this pseudovirus is used to infect two target cell lines that are engineered to express CD4 and either the CCR5 or CXCR4 chemokine co-receptor. Upon completion of a single round of viral replication, successfully infected cells express the luciferase gene carried by the pseudovirus and emit light. R5 viruses will infect the CCR5/CD4 cell line while the CXCR4/CD4 cell line will remain uninfected. Conversely, X4 viruses will infect the CXCR4/CD4 cell line while the CCR5/CD4 cell line will remain uninfected. Pseudoviruses generated from patients with dual or mixed HIV-1 will infect both cell lines. The bioluminescence produced in infected cells is a direct measure of virus entry and is quantified to determine virus tropism. The application of specific co-receptor antagonists during the pseudovirus infection stage of the assay confirms the tropism designation.

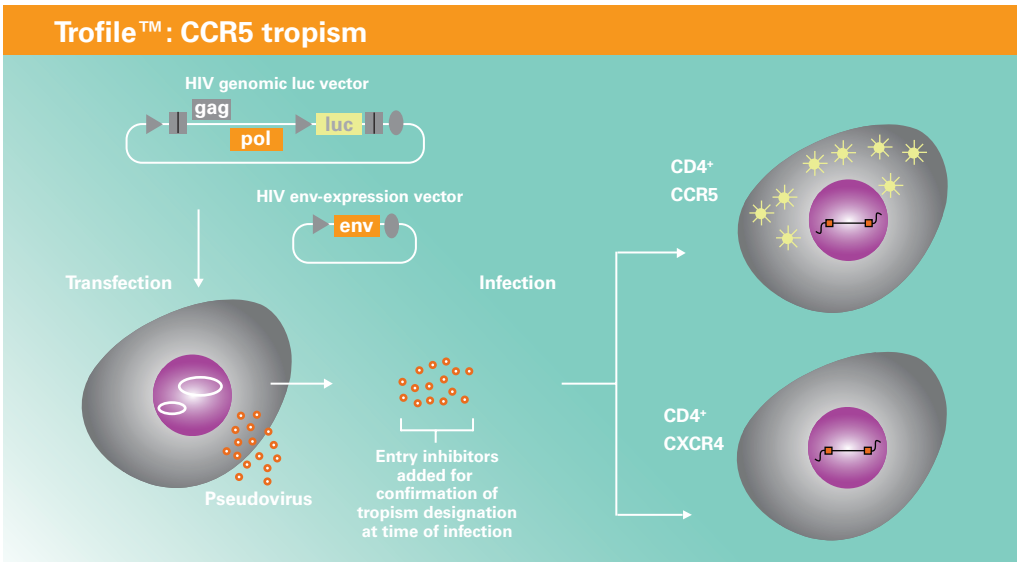


Figure 2. Schematic diagram of the assay. Replication-defective virus particles are produced by cotransfecting HEK-293 cells with the env-expression vector derived from patient's sample and an HIV-1-genomic vector in which the *env* region is deleted and replaced with a luciferase cassette. The recombinant virus particles are harvested and used to infect target cells that express CD4 and either the CCR5 or CXCR4 co-receptor. The R5 virus will be able to infect the cells expressing CCR5 but not the CXCR4-expressing cells. Conversely, an X4 virus will infect the CXCR4-expressing cells but not CCR5-expressing cells. Tropism designation can be confirmed by the addition of specific co-receptor antagonists at attempted infection.[13]

2.2 Advantages of using Trofile

Trofile utilizes the entire envelope region capturing all known determinants of co-receptor tropism.

Trofile is the most sensitive high throughput tropism assay available to clinicians, detecting minority subpopulations of alternate tropism in the range of 5%.[13] Trofile is the most accurate tropism assay available to clinicians. When presented with a panel of 38 diverse strains of HIV of multiple subtypes (R5, X4, or dual/mixed), Trofile was able to correctly identify the tropism in all samples.[14] Over 23,000 samples have been tested using this assay and Trofile is regarded as the gold standard in commercial tropism testing. All clinical trials testing co-receptor antagonists have used Trofile.

2.3 Genotypic analysis of the V3 region to determine HIV-1 co-receptor tropism

The genotypic determinants of HIV-1 co-receptor tropism remain to be defined. Evidence indicates that the majority of tropism determinants are located within the V3 region of the surface envelope protein gp120. Further, it seems that multiple genetic determinants, rather than single genotypic changes, are responsible for viral tropism. Based on these results, several algorithms have been established to try and predict HIV-1 tropism based on genotype.

2.4 Limitations of genotypic analysis of tropism

There are several challenges to employing genotypic approaches in determining HIV-1 tropism.

1. The envelope region exhibits a high degree of sequence diversity and variable length, making sequencing technically challenging. The presence of insertions and deletions, "indels," can lead to inaccurate sequence determinations.

2. Genotypic analysis has limited ability to identify minority HIV-1 populations. Standard genotypic resistance testing can identify minor variants in the population down to 15% to 25%. In comparison, V3 sequencing faces additional challenges than standard genotypic resistance testing because of the presence of indels. This inherent sequence diversity would more than likely reduce the ability of V3 sequence data to identify minority populations.[15]

3. The algorithms currently used to predict co-receptor tropism in clade B HIV-1 are not precise.[15] Subtype specific algorithms for non-clade B HIV-1 will likely be required but have not yet been defined.

4. Finally, not all determinants of tropism are found in the V3 region of the gp120 protein. For example, a recent report showed that a single mutation in the gp41 protein was able to confer an R5 virus with the ability to use the CXCR4 co-receptor (ie, dual-tropic virus) (Figure 2).[16] Thus two viruses with identical V3 sequences could have differing tropisms.

Regions outside the V3 region can modify HIV-1 viral tropism

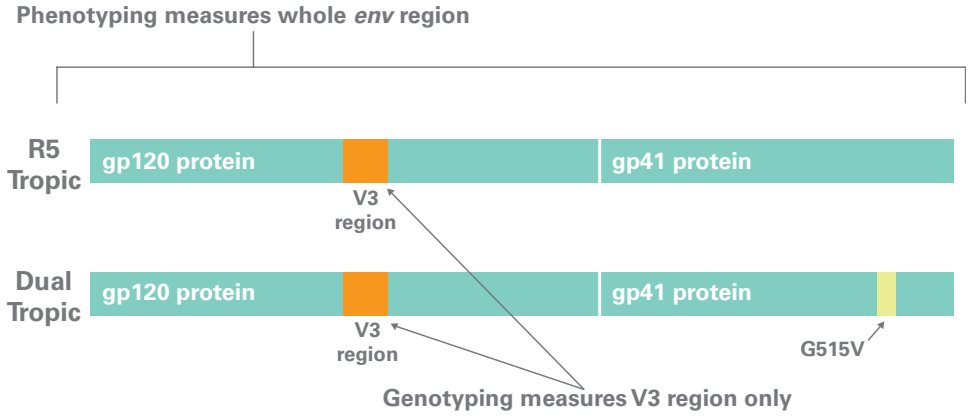


Figure 3. Map of the entire *env* region of two HIV-1 viruses, one that has CCR5 tropism and one that has dual tropism. Both viruses have identical V3 sequences on the gp120 protein. However, a single mutation on the gp41 protein (G515V) was able to confer dual tropism. As this region is outside the V3 region, V3 genotyping could incorrectly designate this virus as CCR5 tropic.

3. Why test before treatment with CCR5 antagonists?

Individualized medicine is built on the concept of devising medical strategies that will be most beneficial to a specific patient. In breast cancer, before Herceptin® is prescribed, the clinician orders a test to see whether the patient's tumor expresses high levels of HER2, a protein that is the target for Herceptin. If the tumor does not express HER2, Herceptin will be of reduced benefit to the patient.[17]

3.1 Know your patients' HIV-1 tropism

The same is true with CCR5 antagonists. These drugs antagonize HIV-1 that utilizes the CCR5 co-receptor to enter host cells. Clinicians testing for HIV-1 tropism in a patient will be able to determine whether the patient's virus is a suitable target for this drug. Patients with other tropism classifications (non-R5 HIV-1) will derive little clinical benefit from CCR5 antagonists, as the virus can continue to enter the host cell using CXCR4 co-receptors not blocked by CCR5 antagonists.[18]

The importance of tropism testing before initiation of CCR5 antagonist treatment was demonstrated in several clinical trials. In the MOTIVATE 1 and 2 trials, the CCR5 antagonist maraviroc was tested in individuals who had R5 viruses.[11,12] The other trial (A4001029) tested the antiviral activity of maraviroc in individuals who had dual/mixed viruses.[18] In the R5 patient population, after 24 weeks of [BID] treatment with the CCR5 antagonist plus optimized background therapy, reductions of –1.95 and –1.97 HIV-1 RNA $\log_{10}$ copies/mL were seen (MOTIVATE 1 and 2, respectively). Those in the placebo arms (optimized background therapy only) saw reductions of –1.03 and –0.93 HIV-1 RNA $\log_{10}$ (Table 2). After 24 weeks of treatment, there were increases of 111 and 102 $CD4^+$ cells/mm^3 in the CCR5 antagonist treatment group with optimized background therapy. The placebo arms (optimized background therapy only) saw increases of 52 and 64 $CD4^+$ cells/mm^3 after 24 weeks.

However, a parallel study (A4001029) using maraviroc in individuals with dual/mixed tropic viruses provided very different results.[18] After 24 weeks of treatment a reduction of –1.20 mean HIV-1 RNA $\log_{10}$ in the maraviroc [BID] plus optimized background therapy arm was seen versus a reduction of –0.97 mean HIV-1 RNA $\log_{10}$ in the placebo arm (optimized background therapy only). There were increases of 62 $CD4^+$ cells/mm^3 in the maraviroc [BID] and optimized background therapy arm as compared with an increase of 36 $CD4^+$ cells/mm^3 in the placebo arm (optimized background therapy only) after 24 weeks. The results demonstrate that those individuals who had dual/mixed viruses did not benefit as much from CCR5 antagonism as did those individuals who had R5 virus.

Table 2. Comparison of efficacy of a CCR5 antagonist with R5 and dual/mixed viruses

Trial Name	Treatment Arm	Antiviral Experience	Baseline Tropism	Baseline Mean HIV-1 RNA, $\log_{10}$ copies/mL (range)	Baseline median CD4 cells/mm^3 (range)	Mean Viral Load Reduction ($\log_{10}$) after 24 weeks	Mean Change CD4 cells/mm^3 after 24 weeks
A4001029[18] (24 week)	OBT + placebo	Experienced	D/M	5.01 (3.7 – 6.2)	99 (2 – 650)	– 0.97	+36
Pfizer	OBT + maraviroc [BID]	Experienced	D/M	5.10 (3.6 – 6.7)	96 (0 – 615)	–1.20	+62
A4001027[12] (24 week)	OBT + placebo	Experienced (N America)	R5	4.84 (3.5 – 6.0)	163 (1 – 675)	–1.03	+52
Pfizer MOTIVATE 1	OBT + maraviroc [BID]	Experienced (N America)	R5	4.86 (3.3 – 6.9)	150 (2 – 678)	–1.95	+111
A4001028[11] (24 week)	OBT + placebo	Experienced (global)	R5	4.89 (3.8 – 7.1)	174 (2 – 545)	– 0.93	+64
Pfizer MOTIVATE 2	OBT+ maraviroc [BID]	Experienced (global)	R5	4.84 (3.0 – 6.2)	182 (3 – 820)	–1.97	+102

OBT = Optimized background therapy
[BID] = Twice-daily dosing of maraviroc
D/M = Dual/mixed virus
R5 = R5 virus
MOTIVATE 1 and 2[11,12] = Maraviroc plus Optimized Background Therapy In Viremic, ART-Experienced Patients Infected With CCR5-Tropic HIV-1 (North America [1] and Global [2])
A4001029[18] = Maraviroc plus Optimized Background Therapy In Viremic, ART-Experienced Patients Infected With D/M HIV-1

Glossary of terms

CCR5	The (**c**ysteine-**c**ysteine) chemokine **r**eceptor **5** is expressed on the surface of several immune cell types
CCR5-tropic	A virus that uses the CCR5 co-receptor exclusively to gain entry to its host cells
CCR5-utilizing virus	Viruses that can use the CCR5 co-receptor to gain entry to its host cell. This group includes R5 viruses and dual-tropic viruses
CXCR4	The (**c**ysteine-**X**-**c**ysteine) chemokine **r**eceptor **4** is expressed on the surface of several immune cells. X refers to any amino acid present between the two cysteine residues
CXCR4-tropic	A virus that uses the CXCR4 co-receptor exclusively to gain entry to its host cells
CXCR4-utilizing virus	Viruses that can use the CXCR4 co-receptor to gain entry to its host cells. This group includes X4 viruses and dual-tropic viruses
Dual-tropic	Viruses that can enter host cells using either the CCR5 or the CXCR4 co-receptor
Mixed-tropic	Mixed-tropic populations of viruses that may contain CCR5-tropic, CXCR4-tropic, and/or dual-tropic viruses
Tropism	The ability of a particular HIV-1 virus to infect a target cell using a specific co-receptor (CCR5 or CXCR4)

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