

The XTrack System: Application and Advantage

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Key Words

XTrack system · Geno2pheno · HIV-1 · Drug resistance interpretation · Maraviroc

Abstract

XTrack, a rapid, cost-effective diagnostic HIV tropism system, yields predictive results and dissects virus mixtures. It is based on the duplexing of patient samples with selective DNA probes, combined with sequence-based analysis and replicative phenotyping for ambiguous samples.

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Introduction

Overall, clinical studies have demonstrated a good agreement between the results of phenotypic tests (Trofile) and genotypic prediction systems (e.g. geno2pheno) for the HIV tropism in the plasma of HIV-1-infected patients. Despite an overall good correlation of tropism test with the clinical response of patients to coreceptor antagonists, several key issues still remain insufficiently answered:

In several trials, a substantial number of patients predicted to harbor a virus with nonresponsive tropism did show a significant clinical response to maraviroc (MVC) treatment [1]. Clinically more important, Trofile, as the

most widely used phenotyping system, has been optimized to identify (and exclude) with highest sensitivity patients with CXCR4-tropic viruses in a given clinical specimen. This sensitivity reaches down to the detection of 0.3% of X4-tropic virus (version ESTA [2–4]). As a consequence, all patients with traces of X4-tropic HIV are excluded from therapy with a coreceptor antagonist. This is current practice although deep-sequencing approaches have demonstrated that low frequencies of this ‘bad virus’ can be identified in almost any patient, irrespective of the stage of infection [5] and therapy response [6, 7].

In response to this potential shortcoming of current tropism determination systems, we have developed a test platform, the ‘XTrack system’, that combines sequence aspects and structural aspects in 3 elements: TrackC, geno2pheno_[coreceptor] (a genotypic approach) and PhenX-R: all samples are first tested with TrackC, those with unclear results are analyzed by geno2pheno and ambiguities by replicative phenotyping with PhenX-R.

Element 1 (TrackC)

This is sufficient for approximately 80% of all analyses. The capillary-based resolution of the DNA-DNA hybridization products between a patient-derived viral cDNA sequence spanning the V3 loop of the viral *env*

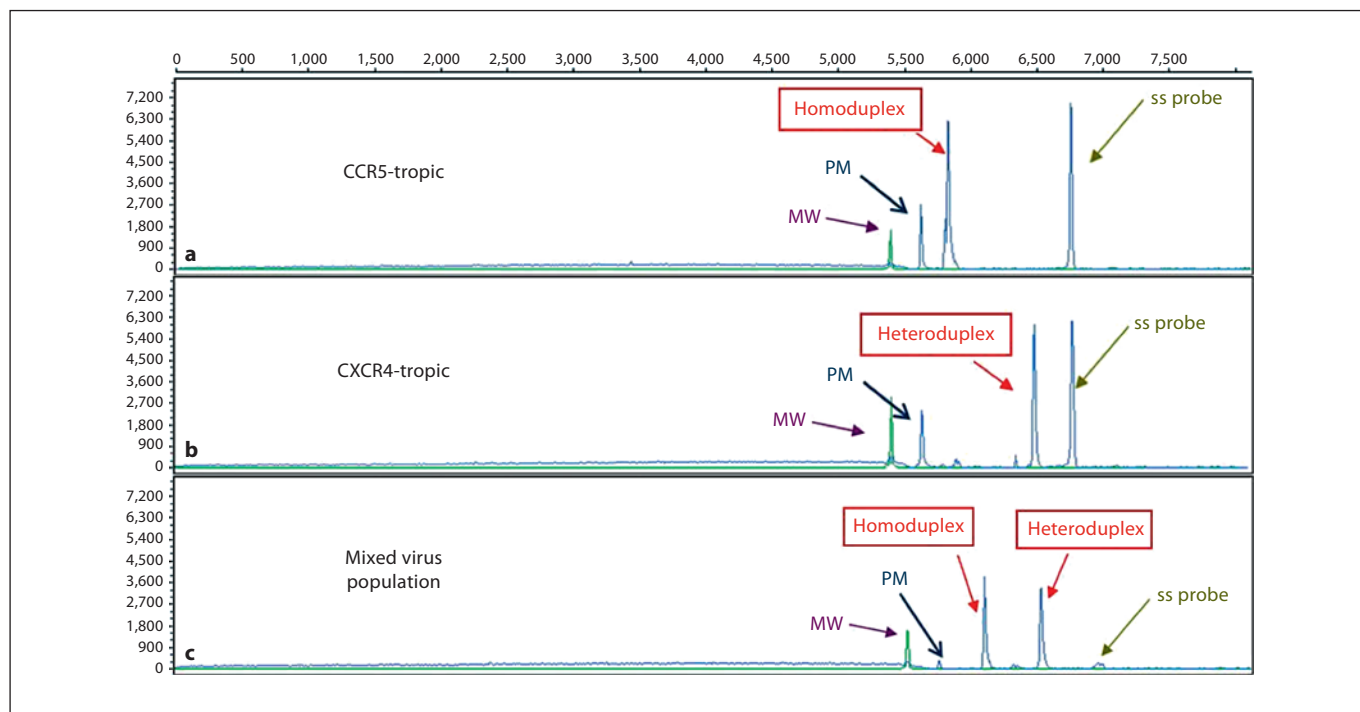


Fig. 1. Three electrophoregrams of the TrackC system for diagnostic tropism determination. Each shows the migration of the single-stranded, fluorescence-labeled V3 probe ('ss probe') as well as a molecular-weight (MW) standard of reference double-stranded DNA. PM = Perfect match of the double-stranded probe. Between these references, the migration of samples as a function of

the relatedness of the R5 probe is displayed. **a** A typical R5-tropic virus. **b** X4-tropic virus with less homology to the probe ('heteroduplex'). **c** Two discrete peaks ('homoduplex', 'heteroduplex') reflect the simultaneous occurrence of both in a patient's sample with a mixed virus population.

gene with a predefined, well-characterized, labeled probe yields migration products that are suitable for separating and classifying R5-tropic and X4-tropic or not-assignable products. Examples are depicted in figure 1.

The use of this simple technique provides answers in the most rapid fashion as the entire process from the workup of viral RNA to the analysis can be fitted into 1 day. TrackC employs 2–3 such V3 probes, and each capillary resolution requires an electrophoretic run of about 40 min. Another advantage of this hybridization step is the ability to easily resolve mixed virus populations: with a sensitivity of 3%, additional virus species or viral minorities are detected and assigned.

We demonstrated that the XTrack system is useful for viral loads as low as 200 copies/ml of plasma and possesses a high technical reproducibility of >95%. It is also applicable to proviral DNA samples [TK, unpublished].

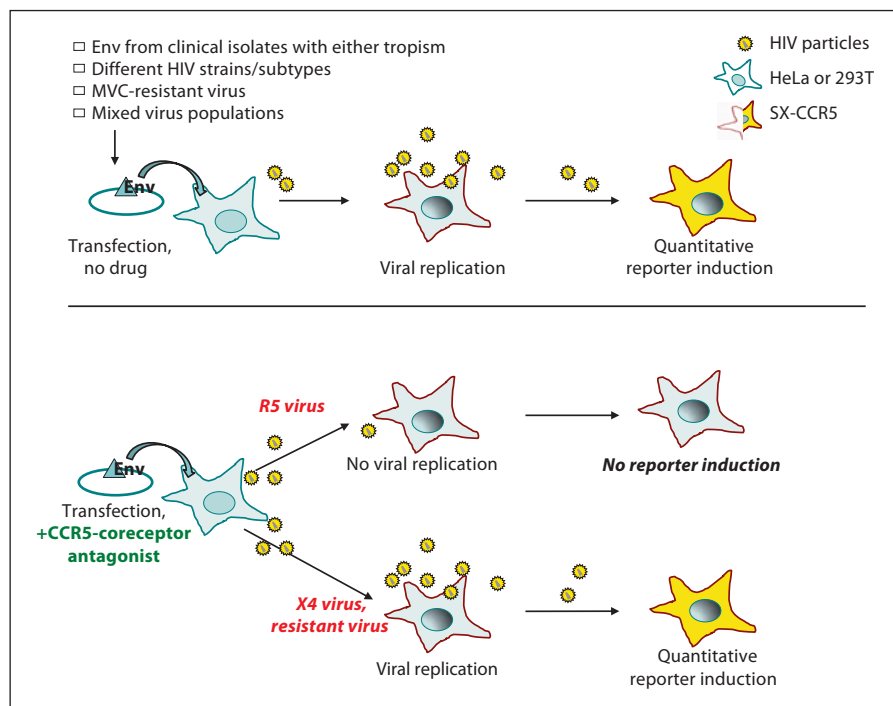
Element 2 (geno2pheno_[coreceptor])

We estimate that in about 20% of analyses a confirmation with a second, independent method is indicated in order to verify the TrackC result. For this second analysis, the nucleic acid sequence of the env V3 region is determined and subjected to a 'classic' genotypic analysis using geno2pheno. In most cases, initial TrackC results can be reproduced; for discordant test results, an algorithm assists with the assignment probability.

Element 3 (PhenX-R)

In rare cases, a tropism assignment can still not be reached with confidence by the first 2 methods since aspects such as length polymorphisms of the V3 loop, the sequence distance of noncanonical HIV variants or the presence of dual-tropic viruses do not permit a straight identification with the genotyping methods.

Fig. 2. PhenX-R is a recombinant, transfection-based phenotyping system. Patient-derived PCR products of the viral *env* are position-precisely inserted into a full-length clone of HIV, thereby reconstituting a replicating viral genome (green circle = Env). Upon DNA transfection into susceptible HeLa cells (with blue lining), the virus multiplies and produces infectious virions (yellow particles). Viruses are used for the subsequent infection of reporter cells (HeLa SXR5, with brown lining), which express both CXCR4 and CCR5 coreceptors and possess an LTR-dependent lacZ gene. In the absence of drugs (upper path) a quantifiable signal correlating with the level of virus expression is produced, independently of the viral tropism. In parallel, the same procedure is performed in the presence of a drug, e.g. MVC. In this case, the quantifiable signal correlates with expression of X4 viruses or R5 viruses resistant to MVC (bottom path). Otherwise the signal remains completely suppressed (path in center).



In these cases, that are estimated to be in the range of <5% of all analyses, PhenX-R, the Swiss phenotypic test system is used. It is directly derived from a similar test system to assess viral resistance to protease inhibitors or reverse transcriptase inhibitors [7]. For this assay, an extended fragment of the viral *env*, spanning at least the V2–V5 region is amplified and faithfully inserted into a viral cassette plasmid lacking the precise region, where it complements a reference HIV-1 genome to restore a fully infectious virus. This replication-competent construct is then transfected into a fully susceptible HeLa cell line that expresses CD4, CCR5 and CXCR4 [8] (fig. 2).

After 4 days and up to 4 rounds of viral replication in the presence of a prototypic coreceptor antagonist, e.g. either TAK779 (blocks CCR5) or AMD 3100 (blocks CXCR4), viral replication is read via the expression or blockade of an HIV-dependent reporter gene stably expressed in these cells. As a consequence, the viral tropism can be read and judged directly along with the level of viral replication as an indicator of viral replicative fitness.

The presence of a replicating virus, although it necessitates a biosafety environment, makes this test very sensitive and also allows the assessment of viral minorities. Moreover, a successive round of infection in the presence of the other inhibitor class precisely separates mixed virus populations from true dual-tropic strains of HIV, and

allows the assessment of viral MVC resistance. This latter aspect has not yet been seen to be clinically relevant, but may become an important factor once coreceptor antagonists come into wider use for patients.

Test Performance

In a parallel analysis of clinical samples from Switzerland and Germany, each without access to the other's results, the performance of the respective elements of this Swiss HIV tropism test system was assessed:

Reportable (for samples claimed to possess $\geq 1,000$ copies/ml), n = 70:

TrackC alone: 85%, TrackC + geno2pheno: 98%, Trofile (ESTA): 68%.

geno2pheno is described in another section of this publication, and the Trofile (ESTA) test is the published and clinically validated version of the Monogram phenotypic system. 'ESTA' stands for 'enhanced sensitivity tropism assay' and is based on the modification of Trofile, with which less than 0.3% CXCR4-tropic viruses in a sample will be detected. The diagnostic service for Trofile is only available through their Californian laboratory.

Ongoing studies follow the analysis of proviral DNA, which will open the application for special indications for

		Samples											
		NRZ01	NRZ02	NRZ03	NRZ04	NRZ05	NRZ06	NRZ07	NRZ08	NRZ09	NRZ10	NRZ11	NRZ12
Subtype		B	F1	AE	A1	D	G	D	C	H	F1	D	AE
Phenotype	culture	NSI	NSI	NSI	SI	SI	NSI	NSI	NSI	-	SI	SI	NSI
	PhenX-R	-	R5	R5	X4	X4	R5	-	-	-	-	X4	R5
	ESTA	-	R5	R5	X4	X4	R5	R5	-	R5	-	-	-
Genotypes n=37 (36 centers)	01	86.1	48.9	13.0	0.5	0.0	42.5	9	38.4	8.5	-	0.1	33.0
	02	86.1	48.9	13.0	0.7	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	03	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	04	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	05	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	06	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	07	-	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	-	0.1	33.0
	08	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	09	90.9	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	-
	11	86.1	48.9	13.8	0.7	0.0	-	8.5	-	8.5	0.7	0.1	33.0
	12	86.1	44.5	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	13	86.1	44.5	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	14	86.1	48.9	13.0	0.5	0.0	42.5	8.5	-	8.5	0.7	0.1	33.0
	15	86.1	52.8	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	16	86.1	44.5	13.0	0.5	-	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	17	86.1	68.6	56.7	0.5	0.0	42.5	8.5	38.4	42.2	1.1	0.2	73.1
	18	86.1	48.9	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	19	86.1	55.3	13.0	-	-	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	20	86.1	48.9	13.0	0.5	0.2	67.0	2.8	38.4	8.5	0.7	0.0	33.0
	21	86.1	44.5	13.0	0.2	0.1	42.5	0.2	38.4	8.5	0.2	0.5	33.0
	22	86.1	44.5	13.0	0.7	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	24	86.1	58.7	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.1	0.1	33.0
	25	86.1	58.7	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	26	86.1	44.5	13.0	0.5	0.0	42.5	8.5	38.4	9.6	0.7	0.1	33.0
	27	86.1	-	13.0	0.5	0.0	42.5	8.5	-	8.5	0.7	0.1	33.0
	28	86.1	-	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	29	-	48.9	-	-	-	42.5	-	-	23.6	0.5	0.1	50.5
	30	86.1	44.5	13.0	0.5	0.0	42.5	8.5	0.1	8.5	0.7	0.1	27.1
	31	86.1	48.9	-	0.5	0.0	42.5	8.5	38.4	8.5	0.5	0.1	-
	32	86.1	30.1	13.0	0.5	0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	33	86.1	44.5	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	34IH	86.1	34.7	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	34RH	86.1	-	-	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	35	86.1	52.8	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	33.0
	36	86.1	34.7	9.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	0.7
	37	86.1	44.5	13.0	0.5	0.0	42.5	8.5	38.4	8.5	0.7	0.1	-

Fig. 3. Compilation of the results of the blinded genotypic tropism testing of 12 clonal HIV-1 variants (NRZ01-NRZ12) by 36 dedicated laboratories (1–36). The resulting HIV tropism reported by the respective centers is given as an FPR value and colored according to the legend. The XTrack system is represented in lane 17. In the upper lanes are phenotypic results determined by 3 different systems: ‘culture’ as MT4-culture/syncytium assay, PhenX-R and ESTA (Trofile).

Color	Usage
Green	genotypic: R5 (FPR ≥ 20%) / Phenotypic: R5
Red	genotypic: X4 (FPR <12.5%) / Phenotypic: X4
Yellow	maybe X4
-	No sequence / No phenotypic result
value	Base calling error

patients with no detectable circulating HIV and also to lower virus loads.

The Swiss system versus Trofile (ESTA), n = 150:
 (for standardization purposes this assessment assumes all Trofile results as ‘true’)

Sensitivity:
 TrackC alone: 76.2%, TrackC + geno2pheno: 85.7%,
 TrackC + geno2pheno + PhenX-R: 98%.

Specificity:
 TrackC alone: 68.6%, TrackC + geno2pheno: 94.3%,
 TrackC + geno2pheno + PhenX-R: 98%.

In the first European Quality Assessment panel [9, 10], the Swiss system was found to match well with the expected outcome (fig. 3). In contrast to the laboratories which provided solely genotype-based results, the findings from Basel yielded 2 discordant results (samples 3 and 9), which, however, agree with the Trofile results. This may reflect the additional properties of the Swiss system that delivered results more in accordance with those from other phenotypic systems. However, it should be noted that the recombinant phenotypic assays could not detect all samples.

The genotypic results from all the participating laboratories were highly concordant. When compared to recombinant phenotypic assays, however, the exclusive use of geno2pheno seems to overestimate X4 viruses. The current false positive result (FPR) cutoffs for geno2pheno are probably too high. FPR cutoffs of 5 or 7.5% would result in R5 prediction for the samples 3 and 9 by most laboratories and be concordant with the Swiss and the phenotypic assays. Further analysis of these samples will follow to elucidate the underlying reasons for the differences in interpretation.

All in all, more extensive prospective use on clinical specimens, along with virological and clinical outcome data, will serve as support in choosing the optimal system for the determination of the HIV tropism prior to using coreceptor antagonists for the treatment of HIV-infected patients.

Conclusion

The XTrack system, although appearing complex, simplifies the HIV tropism analysis by employing:

- A quick duplex-formation assay (TrackC) to assign reliable tropism for the vast majority of samples (>80%) and to reveal mixed virus populations.
- The use of the sequence-based algorithm geno2pheno to confirm or provide further tropism information for samples that remained unassigned by TrackC (approx. 15% of samples).
- Replicative phenotyping (PhenX-R) for functionally assigning the tropism or to identify dual-tropic HIV strains (requires a recombinant virus with sufficient replicative capacity).

Acknowledgments

François Hamy and Vincent Vidal provided critical input to the concept of TrackC. Heinz Stucki and Suzanne Edwards contributed crucially to the validation process of the test system, which was cofunded via the Swiss HIV cohort study, project 521.

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