Video Article

Genotypic Inference of HIV-1 Tropism Using Population-based Sequencing of V3

Rachel A. McGovern[1], P. Richard Harrigan[1], Luke C. Swenson[1]

[1]Laboratory Program, BC Centre for Excellence in HIV/AIDS

Correspondence to: P. Richard Harrigan at prharrigan@cfenet.ubc.ca

Abstract

Background: Prior to receiving a drug from CCR5-antagonist class in HIV therapy, a patient must undergo an HIV tropism test to confirm that his or her viral population uses the CCR5 coreceptor for cellular entry, and not an alternative coreceptor. One approach to tropism testing is to examine the sequence of the V3 region of the HIV envelope, which interacts with the coreceptor.

Methods: Viral RNA is extracted from blood plasma. The V3 region is amplified in triplicate with nested reverse transcriptase-PCR. The amplifications are then sequenced and analyzed using the software, RE_Call. Sequences are then submitted to a bioinformatic algorithm such as geno2pheno to infer viral tropism from the V3 region. Sequences are inferred to be non-R5 if their geno2pheno false positive rate falls below 5.75%. If any one of the three sequences from a sample is inferred to be non-R5, the patient is unlikely to respond to a CCR5-antagonist.

Video Link

The video component of this article can be found at https://www.jove.com/video/2531/

Protocol

Procedure:

1. Extraction:

At least 500 µL of blood plasma is needed as sample input for this genotypic HIV tropism test.

1. Extract HIV RNA from 500µL of plasma using an easyMAG (bioMérieux) automated extractor, or the preferred extraction method of your laboratory.
   Elute the extracted viral RNA into a 60 µL aliquot. Store at -20 degrees Celsius or begin Reverse Transcriptase-PCR immediately after extracting.

2. RT-PCR:

4µL of sample extract will be amplified in triplicate using nested RT-PCR methods. The RT-PCR reaction must be set up in a PCR-clean room.

1. Calculate the amount of reagent required for the number of samples to be amplified. Follow the table below indicating reagent volumes for 1 reaction.

Reagent	Volume (µL) (1X reaction)
DEPC treated water	10.56
2x reaction buffer	20
50% sucrose and 0.04% bromophenol blue mix	4
Forward primer targeting the HIV V3 region	0.32
Reverse primer	0.32
Enzyme - SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase	0.8

Total	36

Table 1. Reagent volumes required for 1 reaction of One-Step RT PCR.
Each one step RT-PCR reaction requires the following recipe:
- 10.56 µL DEPC treated water
- 20 µL of 2x reaction buffer
- 4µL of 50% sucrose and 0.04% bromophenol blue mix
- 0.32 µL of the forward primer targeting the HIV V3 region
- 0.32 µL of the reverse primer
- 0.8 µL of enzyme

For a total of 36µL per reaction.
2. Line up sample extract tubes in a row beside an empty, labeled 96-well PCR plate.
3. Using a repeater pipette, add 36 µL of sample mix to each well of the PCR plate, such that there are 3 wells prepared for each sample extract.
   *Note: This procedure must be performed in triplicate, at minimum, to ensure adequate sampling of the viral population.
4. Using an 8-channel multichannel pipette, add 4µL of sample extract to each of its 3 wells. Change tips between each addition.
5. Cover the wells with PCR strip caps.
6. When transfer is comlete, place the PCR plate in a thermocycler. Set the thermocycler to run with the following program: 30 minutes at 52°C 2 minutes at 94°C 40 cycles of (15 seconds at 94°C, 30seconds at 55°C and 1.5 minutes at 68°C) 5 minutes at 68°C
7. Remove PCR plate from thermocycler. Plate can be stored at room temperature.

Proceed to second round PCR step

3. Second-round PCR:

1. Calculate the amount of reagent required for the number of samples to be amplified. Follow the table below indicating reagent volumes for 1 reaction.

Reagent	Volume (µL) (1X reaction)
DEPC treated water	13.45
60% sucrose, 0.08% cresol red mix	2
Roche HiFi system Buffer 2	2
MgCl$_2$	0.8
dNTP	0.16
Forward PCR primer	0.15
Reverse PCR primer	0.15
Expand HiFi enzyme	0.29
Total	**19**

Table 2. Reagent volumes required for 1 reaction of 2nd round PCR.
Each second round PCR reaction requires the following recipe:
- 13.45 µL DEPC treated water
- 2 µL 60% sucrose, 0.08% cresol red mix
- 2 µL of Roche Hifi system Buffer 2
- 0.8 µL of 25 mM MgCl$_2$
- 0.16 µL of 25 mM dNTP
- 0.15 µL both of the forward and reverse PCR primers
- 0.29 µL Expand HiFi enzyme

For a total of 19 µL per reaction.
2. In a PCR-clean room, using a repeater pipette, add 19 µL of reaction buffer per well to a clean PCR plate.
3. In a post amplification room, using an 8-channel multichannel pipette, transfer 1 µL of the RT-PCR template into the second-round PCR buffer. Change tips between each addition.
4. Cover the wells with PCR strip caps and transfer the second-round PCR plate to a thermocycler.
5. Set the thermocycler to run with the following program: 2 minutes at 94°C 35 cycles of (15 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C) 7 minutes at 72°C
6. Remove plate from thermocycler after the temperature has returned to 25°C.

4. Gel Electrophoresis:

In order to confirm that the V3 region has been successfully amplified, a gel electrophoresis step is performed.

1. Prepare an agarose gel with 0.8 g of agarose powder in 50 mL of 1X TAE Buffer. Stir and melt in microwave for ~1 minute or until agarose is completely dissolved.
2. Add 6 µL of SYBR safe gel stain and swirl to mix.
3. Pour solution into a gel tray, and add enough gel combs to accommodate the number of PCR wells.
4. Once the gel is dry, remove combs and place the gel in the gel apparatus, making sure it is completely submerged in 1X TAE buffer.
5. Using a 10 µL multichannel pipette, add 8µL of each PCR sample to its own well in the gel. Cover the PCR plate after the amplifications have been added to the gel.
6. Add 6 µL of DNA ladder to at least one well per row.
7. Run the gel apparatus at ~100V for ~10 minutes, making sure that the bands do not run off the gel.
8. Place the gel onto a UV trans-illuminator and take a picture of the gel.
   Wells with PCR-amplified DNA will appear light, indicating a successful amplification.
9. Make note of all samples where three amplifications were success. If necessary, repeat RT-PCR for those samples with fewer than 3 amplifications.

Proceed to sequencing

5. Sequencing:

After having amplified viral cDNA, samples can be prepared for sequencing. The sequencing reaction should take place in a post-amplification room.

1. Remove BigDye from the freezer and sequencing primers from the refrigerator. Let the BigDye thaw at room temperature, for no longer than 1 hour.
2. Calculate the amount of reagent required for the number of samples to be run. Follow the table below indicating reagent volumes for 1 reaction.

Reagent	Volume (µL) (1X reaction)
BigDye	0.3
BigDye Buffer	2.1
Primer	2.6
Total	**5.0**

Table 3. Reagent volumes required for 1 reaction of sequencing.
*Note: prepare a separate mix for each primer.
**Note: the BigDye Buffer used in the sequencing reaction can be prepared as follows: Mix 17.5 mL of Trizma hydrochloride buffer solution (1 M) (pH9.0) and 0.125 mL of Magnesium Chloride (1 M). Bring the final volume to 100 mL with reagent grade water. This should be stored at 2-8° C.

3. Using the calculations from 3.3, prepare the sequencing reaction mixes for each primer and vortex for no more than 5 seconds. Aliquot into 0.2mL strip tubes.
4. Aliquot 5 µL of sequencing reaction mix into the appropriate plate wells using a multichannel pipette. Cover the plate(s) containing sequencing reaction mix with a Kimwipe and set aside.
5. Prepare a 1:15 dilution of amplified PCR product by adding 180 µL of Baxter sterile water to the remaining PCR product.
6. Pipette 1 µL of the diluted sample to the bottom of the appropriate plate wells, change pipette tips.
7. When finished transferring sample, seal the plate with strip caps and vortex for 1 second
8. Place the plate in a centrifuge. Set the speed to 145g, when the spin speed reaches 145g, stop the spin.
9. Place the plate in a thermocycler(s) set to the following program: 25 cycles of (10 sec.@ 96° C, 5 sec. @ 50° C, 55 sec. @ 60° C) The volume should be at least 6µL. The cycle should take roughly 1.2 hours.

Proceed to precipitation

6. Precipitation:

To "clean-up" the viral cDNA following the sequencing reaction, perform ethanol precipitation using 95% ethanol.

1. Retrieve plate from the thermocycler and bring to a post-amplification room.
2. Remove strip caps and place them in order on a clean paper towel or Kimwipe.
3. Pipette 4µL of Working EDTA-Sodium Acetate Solution to the side of each sample well. Tap the plate gently so that the EDTA-Sodium solution falls to the bottom of the well. The same tips may be used so long as they do not contact the sample at the bottom of the well.
   *Note: The working EDTA-Sodium Acetate solution can be prepared as follows:
   1. Prepare 3M Sodium Acetate by dissolving 246.1 g of Sodium Acetate in 1 L of reagent grade water. Filter the solution through a 0.45 micro filter and prepare 50 mL aliquots.
   2. Prepare Stock EDTA -Sodium Acetate Solution (1.5 M NaOAc, 250 mM EDTA) by mixing equal volumes of Sodium Acetate (3 M) and solution of EDTA (500 mM) pH 8.0.
   3. Prepare working EDTA-Sodium Acetate Solution by mixing one part stock EDTA-Sodium Acetate Solution with three parts of reagent grade water (10 mL + 30 mL).

4. Pipette 40µL of chilled 95% ethanol onto to opposite side of the sample wells and replace the strip caps.

5. Seal the caps and vortex the plate for 10 seconds. Tap the plate to dislodge any bubbles.
6. Place the plate in the freezer at -20°C for a minimum of 30 minutes and a maximum of 2 hours.
7. a. Once precipitation has occurred, remove the plate from the freezer and place in a centrifuge for 20 minutes at 3700 rpm.
   b. Remove the appropriate amount of Hi-Di Formamide (Applied Biosystems) from the freezer to allow it to thaw before denaturation.

   *Note: Denaturation of a full 96-well plate requires 960µL of HiDi Formamide and thus it is advantageous to prepare ~1.1mL aliquots beforehand.
8. After spinning the plate remove and discard the strip caps. Invert the plate over the waste container (eg. garbage bin) and decant the supernatant. Rid of any remaining supernatant by blotting the inverted plates on paper towel.
9. Fold 2 sheets of paper towel and place on the surface of the plate. Place the plate facedown in the centrifuge and spin at 145g, stopping the spin when it reaches 145g.
10. Remove the plate from the centrifuge and check to be sure wells are empty. Pipette 155µL of chilled 95% ethanol into the wells.
11. Discard the supernatant into the waste container and blot on paper towel and repeat the centrifugation in 4.10.
12. Upon removing the plate from the centrifuge, discard used paper towel and let plate stand face up for 2-5 minutes to allow any remaining ethanol to evaporate.

Proceed to denaturing

7. Denaturing

1. Retrieve the thawed HiDi Formamide and decant into a container large enough for a multichannel pipette,
   *Note: If using pre-aliquoted measures as noted in 6.7b, one tube is required for each 96-well plate to be run.
2. Using a multichannel pipette, distribute 10µL of HiDi Formamide into all wells on the plate, including those that are empty.
3. Cover the plate with a septa mat to form a seal.
4. Place the plate in a thermocycler at 90°C for 2 minutes.
5. Following denaturation, place the plate in a plate holder then load into the sequencer. Upload a prepared sequencing layout. Plates can be kept at -20°C for up to one week before performing the sequencing run.

8. Analyzing the Resultant Data using Base Calling Software.

1. Using ReCall perform automatic base calling with no human intervention, single primer coverage is acceptable. ReCall is a custom base calling software that can be found at the following URL: http://pssm.cfenet.ubc.ca/
   *Note: An account is required to login. To set up an account, click the "Register" button at the login page. Once an account has been created you may access the upload page.
2. When logged in, you may select sample files for upload. Using the browse option you can locate your raw sequencing data for upload with a maximum upload of 20Mb.
   *Note: Web Recall will only accept data files as ABI and SCF files in a .zip, .tar or .tar.gz file
3. Once files for upload have been selected, choose your reference sequence. In this case, be sure the reference sequence is set to 'V3.' You are now ready to click "Process Data."
4. Processed data will appear on the right hand side of the page under the heading "Past Samples." Each run or sample set processed will be placed in a folder labeled with the date. These can be re-labeled by clicking the "Rename" button.
5. Clicking on the folder will bring up a list of samples. Those that are red have failed; those that are green have passed. By clicking on a specific sample and the "View" button, you are able to review the sequence. Follow the instructions on the right side of the page to navigate through the sequence.
   *Note: For this method, it is acceptable to export sequences which pass recall (shown in green) automatically, without manual intervention.
6. If you are satisfied with the results you can choose to download the passing sequences by clicking "Download." Files will be downloaded in a zipped folder.
   *Note: under "Settings" you can select download and email options, including file type.
7. Samples failing to produce clean triplicate sequences should be reamplified in triplicate from extract. If RePCR fails to provide a clean sequence, a minimum of 2 sequences is acceptable for use in tropism inference.

9. Inferring Viral Tropism from Sequences Generated by Population-based Sequencing using the Geno2pheno Co-receptor Algorithm.

1. Sequences can be run through the geno2pheno coreceptor service at the following URL: http://coreceptor.bioinf.mpi-inf.mpg.de/index.php.
2. Samples to upload can be labeled as seen fit. From the drop down menu of the significance setting, select the "optimized cutoffs based on analysis of clinical data from MOTIVATE (2% and 5.75% FPR)"
3. Choose to upload or paste a sequence for analysis, fasta files are acceptable.
4. The geno2pheno FPR can be interpreted as follows: G2P FPR>5.75 is representative of a R5-using viral population; likely to respond to CCR5 antagonists G2P FPR < 5.75 is representative of a non-R5 viral population; unlikely to respond to CCR5 antagonists. G2P <2 is very unlikely to have any response to CCR5 antagonists. If any one of the three sequences from a sample is inferred to be non-R5, the patient is not likely to respond to a CCR5-antagonist.

10. Representative Results

When the protocol is performed correctly one should expect to successfully sequence 2 or 3 replicates for each sample. Negatives run alongside samples should have no indication of RNA. The majority of sequences should "pass" basecalling by ReCall.

Based on the distribution of R5 and non-R5 within the community viral population, the majority of randomly selected patient samples would be expected to have R5-using virus. Therefore unless the project design would suggest otherwise, one should expect to have more R5 than non-R5 samples.

Table 1. A) The reagent volumes required for 1 reaction of One Step RT-PCR and B) the thermocycler program necessary to carry out the RT-PCR reaction.

A)

Reagent	Volume (µL) (1X reaction)
DEPC treated water	10.56
2x reaction buffer	20
50% sucrose and 0.04% bromophenol blue mix	4
Forward primer SQV3F1	0.32
Reverse primer CO602	0.32
Enzyme - SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase	0.8
Total	**36**

*Note:
SQV3F1 primer sequence: 5' GAG CCA ATT CCC ATA CAT TAT TGT 3'
CO602 primer sequence: 5' GCC CAT AGT GCT TCC TGC TGC TCC CAA GAA CC 3'

B)

No. of Cycles	Time	Temperature
1	30minutes	52°C
1	2minutes	94°C
40	15seconds	94°C
	30seconds	55°C
	1.5minutes	68°C
1	5minutes	68°C

Table 2. A) The reagent volumes required for 1 reaction of second round PCR and B) the thermocycler program necessary to carry out the second round PCR reaction.

A)

Reagent	Volume (µL) (1X reaction)
DEPC treated water	13.45
60% sucrose, 0.08% cresol red mix	2
Roche HiFi system Buffer 2	2
$MgCl_2$	0.8
dNTP	0.16
Forward primer SQV3F2	0.15
Reverse primer CD4R	0.15
Expand HiFi enzyme	0.29
Total	**19**

*Note:
SQV3F2 primer sequence: 5' TGT GCC CCA GCT GGT TTT GCG AT 3'

CD4R primer sequence: 5' TAT AAT TCA CTT CTC CAA TTG TCC 3'

B)

No. of Cycles	Time	Temperature
1	2minutes	94°C
35	15seconds	94°C
30seconds	55°C	
1minute	72°C	
1	7minutes	72°C

Table 3. A) The reagent volumes required for 1 reaction of sequencing and B) the thermocycler program necessary to carry out the sequencing reaction.

A)

Reagent	Volume (µL) (1X reaction)
BigDye	0.3
BigDye Buffer	2.1
Primer Forward V3FO2F Reverse SQV3R1	2.6
Total	**5.0**

*Note: DO NOT combine primers; a separate mix should be prepared for each primer
V3O2F primer sequence: 5' AAT GTC AGY ACA GTA CAA TGT ACA C 3'
SQV3R1 primer sequence: 5' GAA AAA TTC CCT TCC ACA ATT AAA 3'

B)

No. of Cycles	Time	Temperature
25	10seconds	96°C
5seconds	50°C	
55seconds	60°C	

Discussion

The method presented here is a standard sequencing method applied to tropism testing. The clinical application of HIV envelope V3 loop sequencing to predict viral tropism has until late been limited. This method has been shown, in retrospective analyses of the maraviroc (ViiV Healthcare) clinical trials, to be at minimum equally able to predict viral tropism in comparison to other validated assays used clinically.

There are many benefits to performing genotypic analysis of the V3 loop for tropism prediction. First and foremost, this procedure can be performed at any facility with operational sequencing equipment, greatly increasing the accessibility and reducing the turnaround time of tropism testing. In comparison, the phenotypic Enhanced Sensitivity Trofile Assay (ESTA) (Monogram Biosciences), which has served as the gold standard for tropism testing, is performed at one centre in Southern California. Additional benefits include the requirement of less starting material and a minimum required plasma viral load of 500copies/mL. As well, the operational costs of running the genotypic assay are relatively low in comparison to those of a phenotypic assay. The use of the ReCall software in particular eliminates the requirement for manual sequence review, which tends to be a labour intensive part of genotype analyses.

The method presented here is fairly straightforward, with no particular step outweighing another in importance. We do suggest running PCR and sequencing in triplicate to increase the odds of capturing minority species within the viral population of a sample. Replicates greater than three can be performed, however we have found triplicates to be both efficient and reliable. We also suggest using the One-Step Reverse Transcriptase PCR Kit (Qiagen) which performs both RT-PCR and first round PCR in the same reaction while maintaining high sensitivity and specificity.

Disclosures

P. Richard Harrigan has conflicts of interest with ViiV Healthcare, Virco, Merck, Abbott, and Quest. This video-article is sponsored by ViiV Healthcare.

Acknowledgements

References

1. Feng, Y., Broder, C.C., Kennedy, P.E., *et al.* HIV-1 entry cofactor: functional cDNA cloning of a seven transmembrane, G protein-coupled receptor. Science 272, 872-77 (1996).
2. Dragic, T., Litwin, V., Allaway, G.P., *et al.* HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381: 667-673 (1996).
3. Dorr, P., Westby, M., Dobbs, S., *et al.* Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob. Agents Chemother. 49, 4721-4732 (2005).
4. Gulick, R.M., Lalezari, J., Goodrich, J. *et al.* Maraviroc for previously treated patients with R5 HIV-1 infection. N. Engl. J. Med. 359, 1429-1441 (2008).
5. Cooper, D.A., Heera, J., Goodrich, J. *et al.* Maraviroc versus efavirenz, both in combination with zidovudine/lamivudine, for the treatment of antiretroviral-naïve subjects with CCR5-tropic HIV-1. J. Infect. Dis. 201:803-813 (2010).
6. Low, A.J., McGovern, R.A., Harrigan, P.R. Trofile HIV co-receptor usage assay. Expert Opin. Med. Diagnostics 3: 181-191 (2000).
7. Sing, T., Low, A.J., Beerenwinkel, N., *et al.* Predicting HIV co-receptor usage based on genetic and clinical covariates. Antiviral Ther. 12: 1097-1106 (2007).
8. Harrigan, P.R., McGovern, R., Dong, W., *et al.* Screening for HIV tropism using population-based V3 genotypic analysis: a retrospective virological outcome analysis using stored plasma screening samples from MOTIVATE-1. Presented at: 5th International AIDS Society Conference. Cape Town, South Africa, 19-22 July 2009, Abstract WELBA101 (2009).
9. McGovern, R.A., Dong, W., Mo, T., *et al.* Optimization of clinically relevant cut-points for the determination of HIV co-receptor usage to predict maraviroc responses in treatment experienced (TE) patients using population V3 genotyping. Presented at: 12th European AIDS Conference. Cologne, Germany, 11-14 November 2009, Abstract PE3.4/8 (2009).
10. Swenson, L.C., McGovern, R.A., Dong, W. *et al.* Optimization of clinically relevant cutoffs for determining HIV co-receptor use by population and "deep" sequencing methods. Presented at: 47th Annual Meeting of the Infectious Diseases Society of America. Philadelphia, PA, USA, 29 October-1 November 2009, Abstract 297 (2009).
11. Swenson, L., McGovern, R., Dong, W., *et al.* Phenotypic screening for HIV tropism versus both population-based and "deep" sequencing. Presented at: 49th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA, 2009 12-15 September (2009).
12. Swenson, L.C., Moores, A., Low, A.J., Thielen, A., Dong, W., Woods, C., Jensen, M.A., Wynhoven, B., Chan, D., Glascock, C., Harrigan, P.R. Improved Detection of CXCR4-Using HIV by V3 Genotyping: Application of Population-based and 'Deep' Sequencing to Plasma RNA and Proviral DNA. Journal of Acquired Immune Deficiency Syndromes 54(5):506-510 (2010).
13. Swenson, L.C., Boehme, R., Thielen, A., McGovern, R.A., Harrigan, P.R. Genotypic determination of HIV-1 tropism in the clinical setting. HIV Therapy 4(3): 293-303 (2010).