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## **Important Note about the OLA kits regarding IBR1 and IBR2 primers**

The amount of IBR1 and IBR2 primers varies from lot to lot. These amounts are not noted on the vials, so please reference the lot number on the vial of these primers to make sure you are using the correct amount to reconstitute to 10 pmole/ul:

Some of Lot 1 of the OLA kits:

IBR1 (Lot 040135) is 1300 pmole/vial

IBR2 (Lot 040136) is 1300 pmole/vial

Lot 2 of the OLA kits:

IBR1 (Lot 050002) is 1375 pmole/vial

IBR2 (Lot 050003) is 1549 pmole/vial

Some of the older kits do not have the IBR1 and IBR2 vials in them and the vials are sent separately with the shipment.

Sincerely,

NIH AIDS *Reagent Program Staff*

**Genotyping Kits for the Detection of HIV-1 *pol* Drug-  
Resistance Mutations by an Oligonucleotide Ligation Assay**

**Protocol Version 1.3**

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## A. Introduction

The following protocols describe PCR conditions for the amplification of the HIV-1 *pol* region and subsequent analysis by the oligonucleotide ligation assay (OLA) for the evaluation of primary mutations associated with drug-resistance to currently available antiretrovirals. For a detailed review of the method's background, please refer to references (1, 2, 3). The current document provides the detailed procedures required to utilize the kit components.

The kits include PCR primers for amplification of HIV-1 *pol*, reference plasmids containing the mutations of interest as well as the wild-type sequence, and the specific oligonucleotides needed for the detection of mutant and wild-type sequences at the codons of interest. Three kits are available, each designed to detect drug-resistance mutations associated with a particular class of antiretrovirals:

**Kit 1:** Detection of resistance mutations to protease inhibitors  
(nelfinavir, saquinavir, indinavir, ritonavir, amprenavir and lopinavir)

Mutations detected: D30N, I50V, V82A, V82S, V82T, I84V, N88D and L90M.  
Control plasmids included: p30N-88D, p50V, p82A, p82S, p82T, p84V, p90M and p8E5.

**Kit 2:** Detection of resistance mutations to non-nucleoside reverse transcriptase inhibitors (nevirapine, delavirdine, efavirenz)

Mutations detected: K103N and Y181C  
Control plasmids included: pNVP and p8E5

**Kit 3:** Detection of resistance mutations to nucleoside reverse transcriptase inhibitors (abacavir, didanosine, lamivudine, stavudine, tenofovir, zalcitabine, and zidovudine)

Mutations detected: K65R, M184V, T215F and T215Y, and Q151M  
Control plasmids included: p65R, p3TC, pRTMC, pMDR1, p151M and p8E5

The kits' components were designed and optimized for HIV-1 subtype B sequences. The reagents have been evaluated on a limited number of non-subtype B specimens. Work is in progress to identify HIV-1 subtype-specific polymorphisms that might interfere with the assay, and appropriate modifications of the affected reagents will be included in later versions of the kits.

## B. Assay Overview.

Oligonucleotide ligation assays (OLA) are rapid, specific and sensitive reactions for the detection of known point mutations (4, 5). Ligation assays are based on the covalent joining of two adjacent oligonucleotide probes by a DNA ligase when they are hybridized

to a complementary DNA template, usually a PCR product. The specificity of the ligation is regulated by three factors: (i) the hybridization of the oligonucleotides to complementary sequences within the template, (ii) the need for these primers to anneal directly adjacent to one another in a 5' to 3' orientation on the target, and (iii) the requirement that the oligonucleotides have two bases perfectly complementary with the target at either side of the junction (4). These characteristics allow non-stringent ligation conditions, which can be used to type multiple nucleotide substitutions in a single assay.

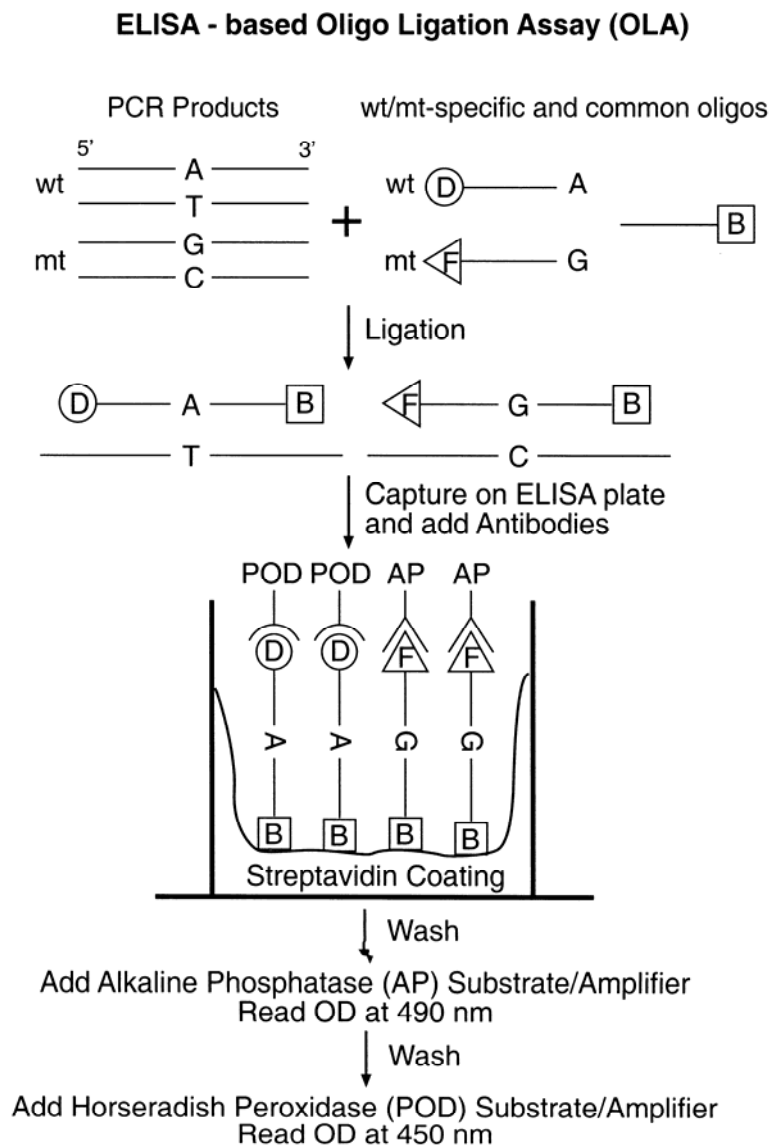
The oligonucleotide ligation assay for the detection of HIV-1 drug-resistance mutations described in this manual utilizes two genotype specific oligonucleotides (a fluorescein and a digoxigenin-modified oligonucleotide specific for the mutant and the wild-type sequence, respectively) and a biotinylated oligonucleotide common to both genotypes. During the ligation reaction, the oligonucleotide probes anneal to their complementary sequence in the PCR product, and the corresponding genotype-specific probe becomes covalently joined to the adjacent common oligonucleotide. The differentially modified ligated products are then captured onto a streptavidin-coated microtiter well, where an ELISA phase of detection using reporter-specific antibodies allows for sensitive and simple detection of both genotypes in a single well (Figure 1 on page 5).

The results of the assay are simple to interpret based on the presence or absence of the color specific for each genotype, magenta for mutant and yellow for wild-type. There are four possible outcomes for a ligation reaction:

- a) Positive mutant, negative wild-type (magenta only); indicates a >95% of mutant sequence in the sample.
- b) Negative mutant, positive wild-type (yellow only); indicates a >95% of wild-type sequence in the sample.
- c) Positive mutant, positive wild-type (magenta and yellow); indicates a mixture of both the mutant and wild-type genotype in the sample.
- d) Negative mutant, negative wild-type (no color); this is considered an indeterminate result and usually indicates the presence of alternative mutations in the sample's sequence within two bases on either side of the ligation site, which interfere with the ligation reaction. In this case, sequencing would be required to determine the patient's genotype at this site.

The results of the assay can be interpreted visually, but readings of the optical densities provide a good tool for record keeping and data analysis. The background in the negative controls and the OD value in the positive controls may vary from assay to assay, therefore the appropriate controls should be run in every assay. Cut-off values for absorbance readings may vary slightly among different plate readers, and will depend on the background obtained on the wild-type and the mutant controls when assayed for the mutant genotype and the wild-type genotype, respectively.

**Figure 1.** ELISA-based oligonucleotide ligation assay (OLA). Following amplification of the HIV-1 *pol* region, PCR products are mixed with two genotype-specific oligonucleotide probes [the mutant (mt)-specific labeled with fluorescein (F), and the wild-type (wt)-specific labeled with digoxigenin (D)] and a biotinylated oligonucleotide common to both genotypes. During ligation, the probes anneal to their complementary sequence in the PCR product, and the genotype-specific oligonucleotide becomes covalently linked to the adjacent common probe. The biotinylated ligation products are captured on streptavidin-coated microtiter wells and an ELISA is performed with alkaline phosphatase (AP) labeled anti-fluorescein antibodies and horseradish peroxidase (POD) labeled anti-digoxigenin antibodies. Sequential addition of the AP and the POD substrates allow for detection of both genotypes in a single well.



## C. Kit Components

### 1) Primers for nested amplification of HIV-1 *pol* from patient specimens:

*First round primers:*

PRA (forward): 5'-CCTAGGAAAAAGGGCTGTTGGAAATGTGG (2011-2039\*)

RTA (reverse): 5'-AACTTCTGTATGTCATTGACAGTCCA (3303-3328\*)

For patient specimens that are not subtype B, replace RTA with:

IBR1 (reverse): 5'-AACTTCTGTATATCATTGACAGTCCA (3303-3328\*)

*Second round primers:*

PRB (forward): 5'-ACTGAGAGACAGGCTAATTTTTTAGGGA (2068-2095\*)

IBR2 (reverse): 5'-CAAAGGAATGGAGGTTCTTTCTGATG (3210-3235\*)

These amplify a 1168-bp fragment spanning from HIV-1 *gag* to codon 228 of the reverse transcriptase gene.

### 2) Primers for amplification of reference plasmids:

PRC (forward): 5'-CTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACT-GTATCC (2201-2242\*)

RT3 (reverse): 5'-TATCAGGATGGAGTTCATAAC (3241-3261\*)

These amplify a 1060-bp fragment spanning from HIV-1 *gag* to codon 237 of the reverse transcriptase gene.

\* Coordinates from the HIV-1 HXB2 genome (GenBank accession no. K03455).

All primers are supplied lyophilized. Before use, **resuspend in TE (10mM Tris/EDTA, pH 8.0) at a concentration of 10pmol/ul and store at -20°C.**

### 3) Control of PCR sensitivity:

DNA from 8E5 cells, which contain one copy of proviral HIV-1 DNA per cell (6), diluted in DNA from uninfected cells at a concentration of 10 copies/0.24ug total DNA/ul.

### 4) Reference plasmids:

HIV-1 mutant and wild-type controls for the OLA were obtained by cloning PCR amplified viral isolates containing the drug-resistance mutations of interest into the

pCR 2.1-TOPO vector (Invitrogen Corporation, Carlsbad, CA). The cloned fragment of each reference plasmid includes nucleotides 2201 through 3261 of the HIV-1 genome. (See Table 1 on page 8 for a description of the reference plasmids.)

Lyophilized plasmid DNA is supplied (5ng total). Before use, **reconstitute in 10ul of TE (Tris-EDTA, pH 8.0) and store at -20°C.**

5) Oligonucleotides for the ligation assay:

Lyophilized oligonucleotides specific for each of the HIV-1 protease and reverse transcriptase codons evaluated are supplied. **Just before use resuspend in 16ul of nuclease free water. Store unused oligonucleotide solutions at -20°C for future use.**

Tables 2 and 3 (pages 9-10) show the nucleotide sequences and modifications of each set of oligonucleotides for the detection of mutations in HIV-1 protease and reverse transcriptase, respectively.



**Table 1. Control plasmids for the OLA**

Plasmid	Reference Genotype	Source	Control for kit #
p8E5	Wild-type	Derived from 8E5/LAV, NIH AIDS Reagent Program	1, 2, 3
p30N-88D	D30N, N88D	Clinical HIV-1 isolate with protease mutations D30N, M36V, A71T, and N88D	1
p50V	I50V	Clinical HIV-1 isolate with protease mutations L10I, K20T, M36I, M46I, I50V, I54M, A71V, and V82I	1
p82A	V82A	Clinical HIV-1 isolate with protease mutations L10F, K20R, M36I, V82A, and L90M <sup>a</sup>	1
p82S	V82S	Clinical HIV-1 isolate with protease mutations L10I, A71V, and V82S	1
p82T-90M	V82T	Clinical HIV-1 isolate with protease mutations K20R, M36I, A71V, V82T, and L90M <sup>a</sup>	1
p84V	I84V	Clinical HIV-1 isolate with protease mutations L10I, A71V, G73S, I84V, and L90M <sup>a</sup>	1
p90M	L90M	Clinical HIV-1 isolate with protease mutations L10I, M46L, I84V, and L90M.	1
pNVP	K103N, Y181C	Derived from HIV-1 <sub>III</sub> B A17 Variant, NIH AIDS Reagent Program.	2
p65R	K65R	Clinical HIV-1 isolate with RT mutations K65R, D123E, D177E, T200I, and I202V.	3
p3TC	M184V	Derived from xxHIV-1 <sub>LAI</sub> -M184V (3TC-resistant), NIH AIDS Reagent Program.	3
pRTMC	T215F	Derived from HIV-1 <sub>RTMC</sub> /MT-2, NIH AIDS Reagent Program, contains RT mutations 67N, 70R, 215F, and 219Q.	3
pMDR1	T215Y	Derived from HIV-1 <sub>RTMDR1</sub> /MT-2, NIH AIDS Reagent Program, contains RT mutations 41L, 74V, 106A, and 215Y.	3
p151M	Q151M	Clinical HIV-1 isolate with RT mutations F77L, F116Y, V118I, Q151M, M184V, and F214L	3

<sup>a</sup> Due to silent mutations present in the vicinity of codon 90, these plasmids should not be used as control for the L90M mutation.

**Table 2. Oligonucleotides used in the OLA to detect mutations in HIV-1 *pol* associated with resistance to protease inhibitors.**

Drug	Codon	Amino acid substitution	Genotype detected	Sequence(5'→3') <sup>a</sup>
Nelfinavir	30	D30N	wt <sup>b</sup> mutant common	dig <sup>c</sup> -TATTAGATACAGGAGCAGATG f <sup>d</sup> -TATTAGATACAGGAGCAGATA p <sup>e</sup> - <b>ATACAGTATTAGAAGAAATGAAT</b> -bio <sup>f</sup>
	88	N88D	wt mutant common	dig-CCTGTCAACATAATTGGAAGAA f-CCTGTCAACATAATTGGAAGAG p- <b>ATCTGTTGACTCAGATTGGTTG</b> -bio
Amprenavir	50	I50V	wt mutant common	dig-AAACCAAAAATGATAGGGGGAA f-AAACCAAAAATGATAGGGGGAG p- <b>TTGGAGGTTTTATCAAAGTAAGA</b> -bio
Ritonavir/Indinavir Lopinavir <sup>g</sup>	82	V82A V82S V82T	wt mutant A mutant S mutant T common	dig-TATTAGTAGGACCTACACCTGT f-TATTAGTAGGACCTACACCTGC f-TATTAGTAGGACCTACACCTAG f-TATTAGTAGGACCTACACCTAC p-CAACATAATTGGAAGAAATCTGT-bio
Amprenavir/Indinavir Nelfinavir/Ritonavir Saquinavir/Lopinavir <sup>g</sup>	84	I84V	wt mutant common	dig-AGGACCTACACCTGTCAACA f-AGGACCTACACCTGTCAACG p- <b>TAATTGGAAGAAATCTGTTGACT</b> -bio
Saquinavir/Nelfinavir	90	L90M	wt mutant common	dig-CAACATAATTGGAAGAAATCTGT f-CAACATAATTGGAAGAAATCTGA p- <b>TGACTCAGATTGGTTGCACTTT</b> -bio

<sup>a</sup> Bases comprising the codons of interest are in boldface type

<sup>b</sup> wt, wild-type

<sup>c</sup> dig, digoxigenin

<sup>d</sup> f, fluorescein

<sup>e</sup> p, phosphate; <sup>f</sup>bio, biotin

<sup>g</sup> mutations associated with HIV-1 resistance to lopinavir in phenotypic susceptibility assays and with virologic failure in clinical trials.

**Table 3. Oligonucleotides used in the OLA to detect mutations in HIV-1 *pol* associated with resistance to reverse transcriptase inhibitors.**

Drug	Codon	Amino acid substitution	Genotype detected	Sequence (5'→3') <sup>a</sup>
Nevirapine Efavirenz Delavirdine	103	K103N	wt <sup>b</sup> mutant common	dig <sup>c</sup> -ACATCCCGCAGGGTTAAAAAAGAAA f <sup>d</sup> -ACATCCCGCAGGGTTAAAAAAGA <b>AC</b> p <sup>e</sup> -AAATCAGTAACAGTACTGGATGTGGGT-bio <sup>f</sup>
Nevirapine Delavirdine	181	Y181C	wt mutant common	dig-ACAAAATCCAGACATAGTTATCTA f-ACAAAATCCAGACATAGTTATCT <b>GT</b> p- <b>TCAATACATGGATGATTTGTATGTA</b> -bio
Tenofovir Zalcitabine	65	K65R	wt mutant common	dig-CTCCAGTATTTGCCATAAAGAA f-CTCCAGTATTTGCCATAAAGAG p- <b>RAAAGACRGTACTAAATGGAGAA</b> -bio
Lamivudine	184	M184V	wt mutant common	dig-AGACATAGTTATCTATCAATACA f- AGACATAGTTATCTATCAATAC <b>G</b> p- <b>TGGATGATTTGTATGTAGGATC</b> -bio
Zidovudine	215	T215F T215Y	wt mutant F mutant Y common	dig-CAACATCTGTTGAGGTGGGGATTTAC f- CAACATCTGTTGAGGTGGGGATTT <b>TT</b> f- CAACATCTGTTGAGGTGGGGATTT <b>TA</b> p-CACACCAGACAAAAAACATCAGAA-bio
All NRTI <sup>g</sup>	151	Q151M	wt mutant common	dig-CAGTACAATGTGCTTCCACA f-CAGTACAATGTGCTTCCA <b>AT</b> p- <b>GGGATGGAAAGGATCAC</b> -bio

<sup>a</sup> Bases comprising the codons of interest are in boldface type

<sup>b</sup> wt, wild-type

<sup>c</sup> dig, digoxigenin

<sup>d</sup> f, fluorescein

<sup>e</sup> p, phosphate; <sup>f</sup>bio, biotin

<sup>g</sup> NRTI, nucleoside reverse transcriptase inhibitors

## D. Reagents Not Included in the Kits

### PCR Reagents:

DNA Polymerase, TAQ Thermus aquaticus (Sigma, D-1806)

10x PCR Buffer with 15mM MgCl<sub>2</sub> (generally supplied with the TAQ enzyme)

2mM dNTP Mix (2mM each dATP, dGTP, dCTP, and dTTP) (Promega, U1240)

Water, Nuclease-Free (Promega, P1193)

Agarose (Ultra Pure) (Invitrogen, 15510-019)

TAE Buffer or TBE Buffer

0.5ug/ml Ethidium Bromide Solution

DNA Molecular Weight Marker

Gel Loading Solution (Sigma, G2526)

### OLA reagents:

Ampligase DNA Ligase, 5U/μl Epicentre Technologies, A32750 or A32250

10x Ligase Buffer                      200mM Tris pH 8.0  
   100mM MgCl<sub>2</sub>  
   10mM DTT  
   Store at -20°C.

10mM NAD                                Sigma, N1511  
   Prepare a 100mM stock solution in dH<sub>2</sub>O,  
   pH to 7.0 with KOH. Aliquot and store at -20° C.  
   Should be stable for at least 1 yr.

BSA Blocking Solution                0.5% BSA in Phosphate Buffer Saline (PBS)  
   Bovine Serum Albumin(BSA), Sigma, A3059  
   Store at 4°C

10X Tris Wash                            1M Tris pH7.5                            For 2L of 10X stock:  
   1.5M NaCl                                242.2g Tris base  
   0.5% Tween 20                            175.2g NaCl  
   Sigma, P1370                                10ml Tween 20  
   Adjust to pH 7.5 with HCl  
   (~100ml) and store at room  
   temperature.

1X NaOH Wash	0.01N NaOH, 0.05% Tween 20 (Prepare a 0.1N NaOH stock solution and dilute before adding the Tween detergent. Make a fresh dilution for each OLA assay)
Antibodies	Anti-fluorescein-alkaline-phosphatase Fab fragments: Boehringer Mannheim, #1426338 (150U)  Anti-digoxigenin-peroxidase Fab fragments: Boehringer Mannheim, #1207733 (150U)
GIBCO Substrate/Amplifier	ELISA Amplification System; Invitrogen, 19589-019 After reconstitution of the lyophilized Substrate and Amplifier, aliquot and store at -20°C.
TMB Substrate	3,3',5,5'-tetramethylbenzidine liquid substrate system Promega, G7431
0.1% Triton X-100	Sigma, T8787. Store at room temperature
0.1M EDTA/0.1% Triton X100	
0.3M H <sub>2</sub> SO <sub>4</sub>	
1M KCl	
Mineral Oil	Sigma, M-5904

Note: As reference we have indicated the companies and catalog numbers we use in our lab, however reagents can be replaced by similar products from a different manufacturer.

#### **E. Equipment/Materials Needed:**

Thermocycler, 96 well

96-well V-bottom Microplates (MJ Research CON9601) or other that fit your thermocycler

96-well Streptavidin-coated Microtiter Plates (Streptawell, transparent, Boehringer Mannheim # 1 734 776)

Microplate Reader, 96-well (if unavailable, assay can be visually interpreted)

Repeating Pipetter

Repeating Pipetter Combitips

Multichannel Pipetter  
Adjustable Micropipettes  
Sterile Aerosol Barrier Pipet Tips  
Microwave Oven  
Agarose Gel Electrophoresis Apparatus  
Power Supply  
UV Transilluminator  
Polaroid Camera

## **F. DNA Preparation**

Manipulation of infected blood should be performed using basic biosafety precautions, and in an environment free of contaminating HIV *pol* DNA. A variety of techniques are suitable for the preparation of HIV DNA, the only requirement is that the subsequent PCR amplification reaction is not substantially inhibited.

An effective method of isolating DNA, involves the isolation of peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque (Invitrogen, Carlsbad, CA) density gradient centrifugation according to the procedure specified by the manufacturer. The DNA is then extracted using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN), or the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. DNA isolated by standard phenol extraction methods is also suitable, as are quick lysis preparations (7).

DNA from dried blood spots collected on filter paper may also be used for the OLA. Depending on the type of filter paper used, DNA elution and purification may be needed prior to PCR (8, 9), or, if using the FTA system (Invitrogen, Carlsbad, CA), PCR may be performed directly on the filter paper after washing inhibitors away (10).

## **G. PCR**

Minor contamination of the starting material can result in wide spread contamination of the PCR amplified products. A common source of contamination is the product of previous amplification reactions or "amplicons". In order to prevent such contamination problems, it is very important to use pipet tips with filters and to separate the "pre" PCR set-up area from the area in which the DNA is amplified, or "post" PCR area. It is best if these two areas are separated physically.

Since every cell sampled is not infected with HIV, nested PCR is required for the preparation of sufficient quantities of HIV *pol* DNA from infected mammalian cells to perform the OLA analysis. Positive and negative control PCR reactions (20 copies of 8E5

cells and reaction mixture without HIV DNA, respectively) should be carried out through both rounds of amplification.

The reference fragments are amplified from 1ng of plasmid DNA by one round of PCR using primers PRC and RT3.

### **Amplification of Patient Specimens:**

Resuspend primers in TE (10mM Tris/EDTA, pH 8.0) at a concentration of 10pmol/ul, as directed on page 6.

#### *First round PCR*

- 1) In the pre-PCR area, prepare the following master mix for one more than the total number of samples, which should include the patient specimens, the 8E5 cells DNA PCR positive control and one or more “water PCR controls” (the latter negative control is to monitor for contamination with PCR product from previous reactions):

For each specimen add:

Nuclease Free Water	33.5 $\mu$ l
10X Buffer	5 $\mu$ l
2mM dNTPs mix	5 $\mu$ l
10pmol/ $\mu$ l PRA	2 $\mu$ l
10pmol/ $\mu$ l RTA*	2 $\mu$ l
<u>TAQ Polymerase (5 U/<math>\mu</math>l)</u>	<u>0.5 <math>\mu</math>l</u>
Total volume	48 $\mu$ l

\*use IBR1 for non-B HIV-1 samples

- 2) Add 48ul of master mix to pre-labeled 0.2 ml PCR tubes, then add 2ul of 8E5 cells DNA (PCR positive control), 2ul of water (negative control), or 1ug of each of the HIV-1 infected DNA samples (in a volume of 2ul) to the corresponding tubes, changing filtered tips between samples. If the patient’s DNA samples are dilute (<1ug DNA in 2ul), adjust the amount of water in the master mix to accommodate the larger volume containing 1ug of DNA (150,000 PBMC); the final volume of the reaction mix should be 50ul.
- 3) Secure all caps tightly on the 0.2 ml tubes and take to the post PCR area. Place tubes into the thermocycler and run the PCR using the following conditions:

<i>Initial denaturing temp.</i>	94°C		
<i>Time</i>	5 min.		
<i>Denaturing temp.</i>	94°C	}	35 cycles
<i>Time</i>	20 sec.		
<i>Annealing temp.</i>	55°C		
<i>Time</i>	20 sec.		
<i>Extension temp.</i>	72°C		
<i>Time</i>	2 min.		
<i>Time</i>	7 min		
<i>Hold temp.</i>	4°C		

### Second round PCR

- 4) In the pre-PCR area, prepare a master mix for the same number of samples as the first round containing the following:

For each sample add:

Nuclease Free Water	33.5 µl
10X Buffer	5 µl
2mM dNTPs mix	5 µl
10pmol/µl PRB	2 µl
10pmol/µl IBR2	2 µl
TAQ Polymerase (5 U/µl)	0.5 µl
<hr/> Total volume	<hr/> 48 µl

- 5) Aliquot 48ul into each second round reaction tube and take to the post-PCR area.
- 6) Transfer 2ul of first round product to the corresponding second round tube, changing filtered tips between samples, and secure caps tightly. Store first round products at -20°C.
- 7) Run the PCR using the following conditions:

<i>Initial denaturing temp.</i>	94°C		
<i>Time</i>	5 min.		
<i>Denaturing temp.</i>	94°C	}	35 cycles
<i>Time</i>	20 sec.		
<i>Annealing temp.</i>	55°C		
<i>Time</i>	20 sec.		
<i>Extension temp.</i>	72°C		
<i>Time</i>	1 min.		
<i>Extension temp.</i>	72°C		
<i>Time</i>	7 min		
<i>Hold temp.</i>	4°C		



- 8) When completed, store samples at -20°C or continue with gel electrophoresis.

### **Amplification of Reference Plasmids:**

Resuspend reference plasmids in TE buffer at a concentration of 0.5ng/ul, as directed on page 7.

- 1) In the pre-PCR area, prepare a master mix for one more than the total number of reference mutant and wild-type plasmids and negative “water” control:

For each sample add:

Nuclease Free Water	33.5 $\mu$ l
10X Buffer	5 $\mu$ l
2mM dNTPs mix	5 $\mu$ l
10pmol/ $\mu$ l PRC	2 $\mu$ l
10pmol/ $\mu$ l RT3	2 $\mu$ l
<u>TAQ Polymerase (5 U/<math>\mu</math>l)</u>	<u>0.5 <math>\mu</math>l</u>
Total volume	48 $\mu$ l

- 2) Aliquot 48ul into each pre-labeled PCR tube and add 2ul of each reference plasmid or water to the corresponding tube.
- 3) Secure caps tightly and take to the post-PCR room. Run the PCR using the same conditions described previously for second round PCR (step 7).
- 4) When completed, store at -20°C or continue with gel electrophoresis.

Note: Because the same wild type reference fragment is used as wild type control for every codon tested, it may be necessary to set up two reactions with the p8E5 plasmid when using kit #1.

### **Agarose Gel Electrophoresis:**

- 1) To analyze second round reactions for amplification product, prepare a 1% agarose gel in 1x TBE or TAE buffer. Load 5  $\mu$ l of each reaction mixed with 1ul of loading dye. Include a lane with DNA Molecular Weight Markers.
- 2) Electrophorese at 80 Volts for approx. 45 minutes.
- 3) Stain the gel in ethidium bromide solution for 20 minutes.
- 4) Distain in distilled water.
- 5) Observe the gel on a UV transilluminator and take a picture for your records.

Notes:

- Agarose gel electrophoresis is conducted to ensure that the correct size DNA fragment was amplified in sufficient amount (i.e. a bright band of the size of the positive control should be visible). Detection of bands that differ from the expected size, or multiple bands, is indicative of either the presence of a substantial population of molecules with internal deletions, or products derived from nonspecific amplification of cellular DNA. These additional products should not interfere with the OLA, as long as the correct size band corresponding to the HIV-1 specific product is present in sufficient amount.
- In some instances, PCR amplification will not yield a product visible on the gel after ethidium bromide staining. This could result from inhibitors of PCR present in the DNA sample, a bad DNA preparation (i.e. the DNA is degraded), or a sample containing a very low HIV-1 DNA load. To increase the yield of the amplification product we recommend repeating the nested PCR with more input DNA or with a new DNA preparation.
- Lack of PCR amplification may also result from the presence of polymorphisms in the patient's HIV-1 nucleic acid sequence in the region complementary to the PCR primers. In this case, we recommend attempting PCR amplification with a different set of primers if available. Any set of primers that will amplify a fragment spanning codon 30 in the HIV-1 protease gene to codon 215 in the reverse transcriptase gene will be suitable (remember to optimize the PCR conditions for this new set of primers).

## H. Oligonucleotide Ligation Assay (see Figure 2 on page 21)

### *Set up ligation plate*

- 1) Use a 96-well V-bottom plate that fits your thermocycler.
- 2) Add 2  $\mu$ l of second round PCR product (*pol* amplicon) from each patient specimen to each of two wells. (Up to 43 patients can be tested in one 96-well plate. We strongly recommend testing samples in duplicate as this increases the chances of detecting errors such as splashing or skipping wells.) Include also two wells containing distilled water (negative controls), two wells with PCR product from p8E5 (wild type controls) and two wells with PCR product from the reference plasmid containing the mutation to be tested (mutant controls). Leave four wells without any sample; these will be used later to blank the spectrophotometer. (See example OLA worksheet on page 22).
- 3) Using a repeating pipetter, add 10ul of 0.1% Triton X-100 to each well.
- 4) Prepare one ligation mix for each codon to be tested, containing the following:  
(Note: Resuspend lyophilized oligonucleotides as directed on page 7)

For a full plate of samples prepare 1200ul of mix:

10X Ligase Buffer	240µl
10mM NAD	240µl
1M KCl	30µl
0.1% Triton X-100	690µl
Wild-type oligo	8µl
Mutant oligo	8µl
Common oligo	8µl
Ampligase DNA ligase	4µl

(If not setting up a full plate of samples, prepare one half or one quarter of the recipe **keeping the proportion of all the reagents**. Store unused oligonucleotides at -20°C for later use.)

- 5) Using a repeating pipetter, add 10µl of the ligation mix to each well.
- 6) Using a 12-channel or a repeating pipetter, add 25µl of mineral oil to each well to prevent evaporation.

***Ligation Reaction:***

- 1) Place the plate in the thermocycler and cover with plastic wrap to prevent contamination of the thermocycler's cover.
- 2) Perform the ligation using the following conditions:
  - 93° C for 30 seconds
  - 37° C for 4 minutes
  - repeat for 10 cycles.

***Stop ligation:***

- 1) Within 10 minutes after completion of the last ligation cycle, add 10µl of 0.1M EDTA/0.1% Triton X-100 to each well using a repeating pipetter.

***Capture on ELISA plate:***

- 1) Transfer each sample from the ligation plate to a streptavidin-coated 96-well microtiter plate using a multichannel pipetter set to 100-150µl.
- 2) Let plates sit for a minimum of one hour at room temperature.

Note: Instead of using commercial streptavidin-coated plates, these plates can be made by adding 50ul of a 25ug/ml streptavidin solution to each well of a 96-well flat bottom microtiter plate and incubating at 4°C overnight. To block this ELISA plate, while the ligation reaction is in progress, empty the streptavidin solution from the plate and blot it on paper towel until dry. Add 200ul of BSA blocking buffer to each well and let the plate sit for a minimum of 30 minutes at room temperature. Before transferring the ligated products to the ELISA plate, empty the blocking buffer and

wash the plate once with 1x Tris Wash. Blot on paper towels until nearly dry, and proceed with step 1 described above.

***Add antibodies***

- 1) Prepare a 1:1000 dilution of both anti-digoxigenin-POD and anti-fluorescein-AP together in the same tube of BSA Blocking Buffer. (Need 5 ml for a full plate.)
- 2) Empty ligation reaction from the ELISA plate and blot on paper towel.
- 3) Wash the plate twice by flooding with 1X NaOH Wash, blotting on a new paper towel after each wash.
- 4) Wash the plate twice with 1X Tris Wash, blotting on paper towel after each wash.
- 5) Add 50µl of antibody dilution to each well using a repeating pipetter.
- 6) Let plates sit at room temperature for 30 minutes.
- 7) During this time, reconstitute the GIBCO substrate and amplifier, or remove previously reconstituted product from the freezer (-20°C) and bring to room temperature for the next step.

***Amplification of reaction:***

*Detection of the mutant genotype:*

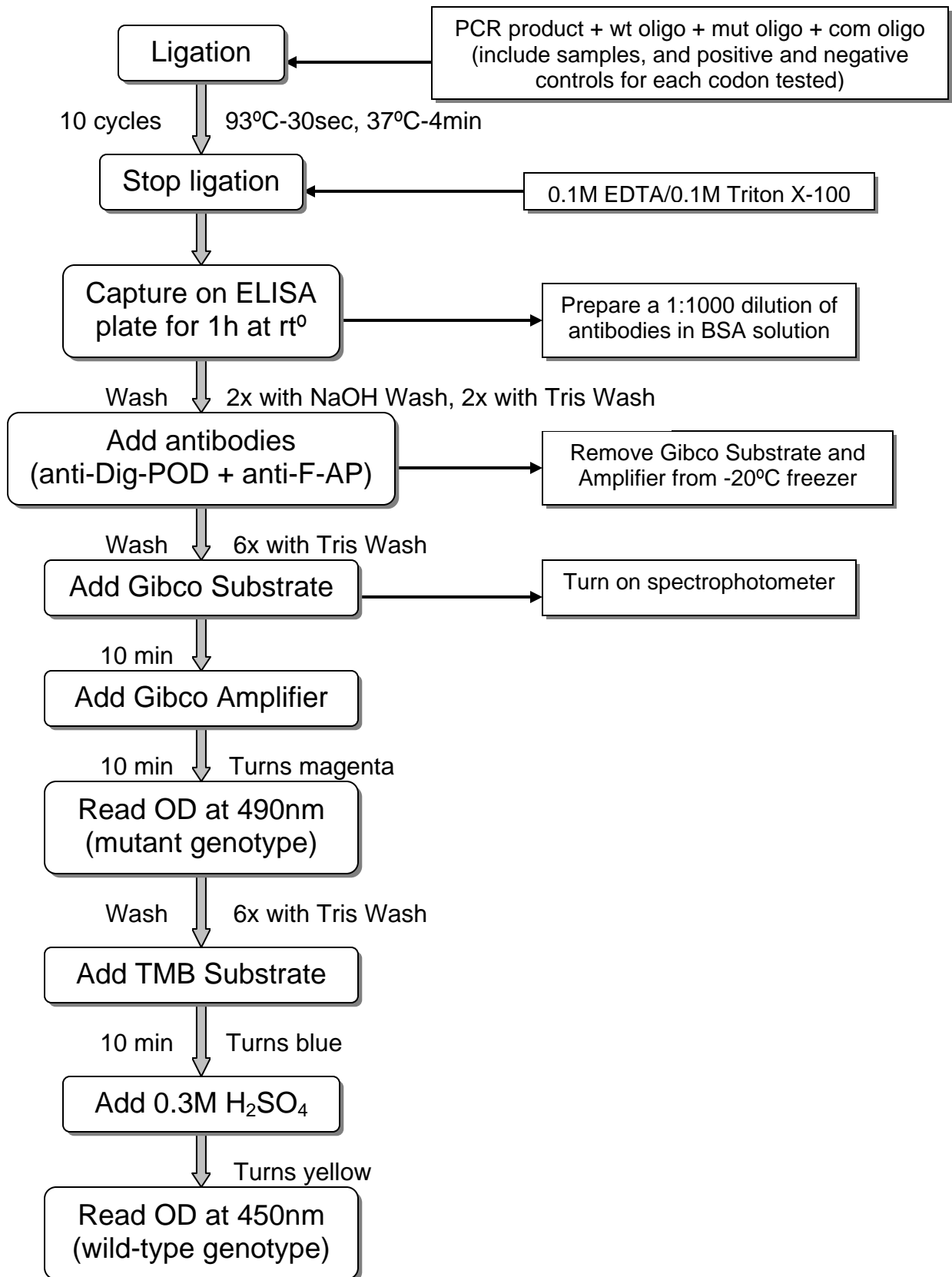
- 1) Wash the plate 6 times by flooding with 1X Tris Wash, blotting on paper towel after each wash.
- 2) Using a repeating pipetter, add 25µl of GIBCO substrate to each well.
- 3) Wait 10 minutes. During this time, turn on the spectrophotometer (plate reader) to warm up.
- 4) Add 25µl of GIBCO amplifier to each well, protect from light, and wait approximately 10 minutes or until the color (magenta) is full in the positive controls but the background in the wild-type control wells is not too dark. (Note: if room temperature in your lab is higher than 22°C, observe the plate soon after addition of the reagent as the color might develop much faster)
- 5) Using the four wells that do not contain ligation product to blank the spectrophotometer, read the plate at 490nm.

*Detection of the wild type genotype:*

- 6) Wash the plate 6 times with 1X Tris Wash by flooding, blotting on paper towel after each wash.
- 7) Add 50µl of TMB substrate to each well.

- 8) Wait approximately 10 minutes or until the color is full in the wild-type positive controls. Color will start to appear quickly and then get darker.
- 9) Once the blue signal is strong but while the background in the mutant control wells is still faint, add 50 $\mu$ l of 0.3 H<sub>2</sub>SO<sub>4</sub> to each well (the color will turn yellow).
- 10) Read the plate at 450nm.

**Figure 2: Oligonucleotide Ligation Assay (OLA)**



## OLA Worksheet

Date: \_\_\_\_\_

Performed by: \_\_\_\_\_

Codon(s) Tested: K103\_\_\_\_\_

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	Blank	Blank	Blank	Blank	H <sub>2</sub> O	H <sub>2</sub> O	p8E5	p8E5	pNVP	pNVP	S1	S1
<b>B</b>	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6	S7	S7
<b>C</b>	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12	S13	S13
<b>D</b>	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18	S19	S19
<b>E</b>	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24	S25	S25
<b>F</b>	S26	S26	S27	S27	S28	S28	S29	S29	S30	S30	S31	S31
<b>G</b>	S32	S32	S33	S33	S34	S34	S35	S35	S36	S36	S37	S37
<b>H</b>	S38	S38	S39	S39	S40	S40	S41	S41	S42	S42	S43	S43

Note: This is an example setup for a full 96-well plate to screen for the mutation K103N in the HIV-1 reverse transcriptase. p8E5 and pNVP indicate the PCR amplified reference fragments for the wild-type and the mutant controls, respectively. S1 through S43 indicate PCR product from patient's specimens 1 through 43. The ligation mix for this plate should include oligos 103 mutant, 103 wild-type and 103 common (total volume is 1200ul).

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**Evaluation of OLA Controls**

Date: \_\_\_\_\_

Performed by: \_\_\_\_\_

Laboratory Name and Address:

\_\_\_\_\_  
 \_\_\_\_\_  
 \_\_\_\_\_

Control	Codon – Amino acid	OD at 490nm (mut detection)		OD at 450 nm (wt detection)	
		1 <sup>st</sup> duplicate	2 <sup>nd</sup> duplicate	1 <sup>st</sup> duplicate	2 <sup>nd</sup> duplicate
p8E5	103 - K				
pNVP	103 - N				
p8E5	181 - Y				
pNVP	181 - C				
p8E5	65 - K				
p65R	65 - R				
p8E5	215 - T				
pRTMC	215 - F				
p8E5	215 - T				
pMDR1	215 - Y				
p8E5	184 - M				
p3TC	184 - V				
p8E5	151 - Q				
p151M	151 - M				
p8E5	30 - D				
p30N-88D	30 - N				
p8E5	50 - I				
p50V	50 - V				
p8E5	82 - V				
p82A	82 - A				
p8E5	82 - V				
p82S	82 - S				
p8E5	82 - V				
p82T-90M	82 - T				
p8E5	84 - I				
p84V	84 - V				
p8E5	88 - N				
p30N-88D	88 - D				
p8E5	90 - L				
p90M	90 - M				

Note: Please fill in the experimental OD values obtained in your lab for the controls included in the kit(s) you received and return to Ingrid Beck by fax: 206-987-7311, or mail:

307 Westlake Ave N, Suite 300; Seattle, WA 98109.