

TDM APV / FOS-APV (ELISA AMPRENAVIR / FOS-AMPRENAVIR)

1. INTENDED USE.....	2
2. PRINCIPLE OF THE TEST.....	2
3. TEST PERFORMANCES.....	2
4. LIMITATIONS	3
5. MATERIALS SUPPLIED.....	3
6. COMPOSITION OF SUPPLIED MATERIALS	3
7. MATERIALS REQUIRED BUT NOT PROVIDED	3
8. STABILITY AND STORAGE.....	4
9. PREPARATION OF REAGENTS	4
10. COLLECTION, STORAGE AND PREPARATION OF SAMPLES	5
11. ASSAY PROCEDURE	5
12. CALCULATION OF RESULTS	6
13. PRECAUTIONS.....	6

1. INTENDED USE

The TDM APV (ELISA AMPRENAVIR / FOS-AMPRENAVIR) is a competitive colorimetric enzyme immunoassay for the quantitative detection of Amprenavir concentration in human plasma samples as an aid in managing patient therapy and monitoring compliance.

This kit can be used also for the determination of Amprenavir in patients treated with Fosamprenavir. In fact, Fosamprenavir pro-drug given “per os” is quickly and almost completely hydrolysed to Amprenavir at the intestinal mucosa level before reaching systemic circulation.

For research use only.

2. PRINCIPLE OF THE TEST

The ELISA Amprenavir / Fos-Amprenavir test is based on the competition between the drug in the plasma sample and the drug conjugated to the detection enzyme; they compete for the same binding sites of an anti-Amprenavir polyclonal antiserum.

A specie-specific solid phase (wells of a microplate) binds the specific antibodies.

A washing step is used to wash away every unspecifically-bound material from the solid phase. The detection of the binding between the enzyme-conjugated drug and the specific antibody is done by the addition of a chromogenic substrate.

The enzymatic activity produces a coloured solution whose absorbance can be read by a microplate reader; the absorbance value is inversely correlated to the drug concentration in the sample.

3. TEST PERFORMANCES

The system is designed for the quantitative detection of Amprenavir in human plasma.

- Dynamic range is from 0.6 to 5 micrograms of drug per millilitre of plasma.
- Accuracy is $\pm 20\%$
- Inter-assay variability calculated on 3 samples tested in 5 different sessions is shown in the following table.

Sample	Conc. session 1	Conc. session 2	Conc. session 3	Conc. session 4	Conc. session 5	Mean conc.	Std. Dev.	C.V.
1	0,53	0,551	0,544	0,505	0,659	0,56	0,06	10,62
2	2,92	3,056	2,793	3,151	2,761	2,94	0,17	5,70
3	1,164	1,248	1,143	1,244	1,214	1,202	0,05	4

- Inter-batch variability calculated on StO analysed by 3 different lots is shown in the following table.

	O.D. batch 1	O.D. batch 2	O.D. batch 3	Mean	Dev.std.	C.V.
St 0	1,24	1,17	1,3	1,24	0,07	5,3

- Intra-assay variability calculated on 3 samples tested in triplicate is shown in the following table.

Sample	O.D. 1	O.D. 2	O.D. 3	Mean O.D.	Std.Dev.	C.V.
1	1,512	1,44	1,512	1,49	0,04	2,8
2	1,336	1,39	1,38	1,37	0,03	2,1
3	0,585	0,546	0,54	0,56	0,02	4,4

4. LIMITATIONS

- No significant cross-reactivity (greater than 0.01% expressed as ratio between the IC₅₀ of the drugs) has been identified to other protease inhibiting drugs commonly used in HIV-1 therapy. Tested drugs include Nelfinavir, Ritonavir, Saquinavir, Indinavir and Lopinavir at therapeutic concentrations.

5. MATERIALS SUPPLIED

Contents	Quantity
Antibody-coated microtiterplate (anti-rabbit IgG)	12x8 wells
Anti-Amprenavir antiserum	1x 12ml
Amprenavir-horseradish peroxidase conjugate (APV- conjugate)	1x 10ml
Sample Diluent	1x 50ml
Amprenavir Calibrators/Standard curve (0, 3.12, 6.25, 12.5, 25, 50 e 100 ng/ml)	7x 0.15ml
TMB 10X	1x 3ml
Development Solution	1x 30ml
Washing Solution 10X	1x 100ml
Stop Solution	1x 7ml

6. COMPOSITION OF SUPPLIED MATERIALS

Antibody-coated Microtiterplate

12 x 8-well strips, coated with anti-rabbit (sheep) antibodies, sealed under vacuum in a polyethylene pouch.

Anti-Amprenavir Antiserum

One vial containing 12ml of anti-Amprenavir rabbit antibodies, in a buffered solution containing a preservative.

Amprenavir-horseradish peroxidase Conjugate

One vial containing 10ml of Amprenavir-horseradish peroxidase conjugate, in a buffered solution containing a preservative.

Sample Diluent

One bottle containing 50ml of a buffered solution.

Amprenavir Calibrators/Standard Curve

7 vials, each containing 0,15 ml of Amprenavir in a buffered solution. Calibrator values are indicated on vial labels. Calibrators are ready-to-use.

TMB 10X

One non-transparent vial containing 3 ml of 10X TMB – buffered solution.

Development Solution

One vial containing 30 ml of a buffered solution.

Washing Solution 10X

One bottle containing 100 ml of a 10X buffered solution.

Stop Solution

One vial containing 7 ml of 1M sulphuric acid solution.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- Calibrated EIA reader adjusted to read at 450 and 620nm; proper software for quantitative ELISA test management is strongly suggested

- Precision pipettes (volumes between 10 and 1000 μ l, \pm 5% accuracy) with disposable tips
- Multichannel 8 tips pipette (volumes 100 μ l \pm 5% accuracy)
- Deionised water
- Vortex shaker
- Microcentrifuge for 1.5ml Eppendorf tubes (10.000 x g)
- 1.5ml Eppendorf tubes
- Reservoirs (disposable) for multichannel pipette
- 1 litre glass cylinder
- 10 ml pipette and pipette-handler
- Timer (60 min. range)
- Test tube racks (for 50 and 1.5ml tubes)
- Filter paper and aluminium foil
- Disposable gloves

8. STABILITY AND STORAGE

- The kits should be stored at 2-8°C and used before the expiration date printed on the box label.
- Shipment should be done at controlled temperature (2-8°C); exposure to temperature up to 30°C for a short period of time (less than 6 hours) doesn't affect device performances.
- Do not freeze.
- Expiration date is printed on the box label; usually, the shelf life is up to 9 months from the manufacturing date.
- After the first use of the reagents, the kit should be properly stored at a 2-8°C and used within 1 month.
- Diluted washing buffer should not be stored for more than 4 hours and must be discarded after use.
- Substrate should be freshly diluted and not reused.
- Samples should not be stored for more than 4 hours after dilution.

9. PREPARATION OF REAGENTS

- If the kit is not to be used completely, remove only the number of strips being used for the day's testing and place the remainder at 2-8°C in foil pouch sealing it carefully with a Scotch tape.
- Take only aliquots of the reagents provided, leaving the bulk in the original vial and place the remainder immediately at 2-8°C. For the Calibrators use sterile tips.
- Bring all reagents to room temperature 30' before use.
- Prepare the required volume of washing solution by diluting 1:10 the Washing Solution 10X with deionized water in the glass cylinder.
- Prepare the required volume of chromogen solution by diluting 1:10 the TMB 10X with the Development Solution in a clean container.
Warning: dilute the substrate just before use (after washing the plate) and keep away from direct light.
- Always wear disposable gloves.

10. COLLECTION, STORAGE AND PREPARATION OF SAMPLES

- The device is designed for the analysis of human plasma.
- No patient preparation is required for blood withdrawal.
- Qualified personnel using approved aseptic venipuncture techniques should collect the blood sample.
- It is important to preserve chemical and biological sample integrity till the analysis procedure.
- *Warning: hemolysed samples are not suitable to be tested by this procedure.*
- Do not add preservatives to the samples.
- *Warning: sodium azide is a strong inhibitor of horseradish peroxidase and should NOT be used.*
- Samples should be stored at 2-8°C and analysed within 24 hours. For prolonged storage, freeze samples and store frozen at -20°C.
- Avoid repeated freeze-thaw cycles.
- Carefully thaw samples if stored at -20°C, mix well and spin them for 5 minutes at 10.000 x g.
- Dilute each plasma sample 1:100 with Sample Diluent; prepare dilutions (10µl of plasma in 990µl of diluent) in Eppendorf tubes. Mix well by inversion or use a Vortex mixer.
- *Warning: it has been noticed that in patients treated with Fosamprenavir plasma levels of Amprenavir are generally more elevated than in patients treated Amprenavir. In this case, as for any sample with concentrations > 5µg (up to 10 µg), it is possible to dilute samples at 1:200 (dilute 100 µl of the first dilution 1:100 with additional 100 µl of Sample Diluent). To obtain the final concentration value the measurements will then be multiplied by a dilution factor of 200.*
- Samples should be tested in duplicate.
- Always wear disposable gloves.

11. ASSAY PROCEDURE

- Use a proper experimental microplate sheet to record sample positions.
- Samples, blanks and standard curve (7 points) should be tested in duplicate.
- Transfer 20 µl of finally diluted samples and Calibrators into the proper wells.
- Change pipette tip between samples.
- Dispense 80 µl of Amprenavir-horseradish peroxidase conjugate in all wells, except for the blank wells, using a multichannel pipette.
- Dispense 100 µl of Anti-Amprenavir rabbit antiserum in all wells, except for the blank wells, using a multichannel pipette.
- Incubate for 60 min. at room temperature (RT).
- Wash the wells 5 times with 350 µl/well of 1X Washing Solution. If washing is done manually, empty the plate by shaking out the liquid and blotting inverted on a paper towel.
- Add 200 µl of diluted chromogen solution in each well using the multichannel pipette.
- Incubate for 30 min. at room temperature (RT) in the dark
- Add 50 µl of Stop Solution in each well using the multichannel pipette.
- Read the absorbance at 450 nm by a microplate reader.

Warning 1: colour development can be monitored by using a 620 nm filter before adding the Stop Solution.

Warning 2: final absorbances should be read within 10 minutes after addition of Stop Solution.

- The test is valid if the following parameters are met:
 - Mean absorbance value of 0 Calibrator higher than 0,8 O.D.
 - Mean absorbance value of Blank lower than 0,2 O.D.
 - IC₅₀ (drug concentration value that inhibits absorbances at 50%) between 10 and 20ng/ml

12. CALCULATION OF RESULTS

- If calculations are made by an ELISA software, use a 4-Parameter Logit-Log method.
- If calculations are made manually, for every well calculate B/B₀ value. B/B₀ is expressed as follows:

$$\frac{\text{Mean absorbance of Calibrators or Samples} \times 100}{\text{Mean absorbance of 0 Calibrator}}$$

Using a semi-log chart paper, assign concentration values of Calibrators (as printed on labels) to the X-axis and B/B₀ values to the Y-axis. Draw the standard curve and interpolate on it the concentration values for samples.

Warning: calculated concentration values must be multiplied by the dilution factor of samples (x 100) to obtain concentration in plasma.

Samples with concentrations greater than 5µg (up to 10 µg) can be diluted 1:200 (dilute 100 µl of the first dilution 1:100 with additional 100 µl of Sample Diluent). In order to obtain the final concentration, the measured values must be multiplied by a dilution factor of 200. This higher dilution can also be useful in testing samples from patients treated Fosamprenavir

13. PRECAUTIONS

- For research use only.
- This device is designed to be used by properly trained laboratory personnel.
- Device and its components must be disposed of according to current legislation.
- Device does not need to be deactivated.
- Always wear disposable gloves when using the device.
- In case of ingestion or contact with eyes, skin or mucosae, wash with plenty of water and consult a physician.
- If the kit is not to be used completely, remove only the number of strips being used for the day's testing and place the remainder in foil pouch with desiccant at 2-8°C.
- Take only aliquots of the reagents provided, leaving the bulk in the original vial.
- For reagents to be dispensed by Multichannel pipette, increase the volume by 1 ml.

TDM APV / FOS-APV (ELISA Amprenavir / Fosamprenavir)

96 wells, code 1678

Rev.: 3

Date of release: 11.07.2005