firstFlagX®

firstFlagX - Androgen Bioactivity Test Kit

92 Tests for veterinary in vitro diagnostic purposes

Catalogue Reference: **FFX-1**

User Manual

Version 2.0

FOR MEDICINAL PRODUCTS / VETERINARY MEDICINAL PRODUCTS / MEDICAL DEVICES FOR MEDICAL AND VETERINARY PURPOSES / ACTIVE IMPLANTABLE MEDICAL DEVICES / IN VITRO DIAGNOSTIC MEDICAL DEVICES FOR MEDICAL AND VETERINARY PURPOSES / LABORATORY REAGENTS / COSMETIC PRODUCTS ONLY

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1. INTENDED USE

The assay is an in vitro diagnostic for equine veterinary purposes and is designed to screen plasma samples for androgen bioactivity (i.e., whether samples contain compounds that activate the androgen receptor [AR]).

2. PROTOCOL SUMMARY

Reconstitute Tubes A, B, C & D as instructed. Aliquot volume needed for number of samples into new tubes and keep in cool caddy. Freeze the remaining Tubes A, B, C & D as instructed. Prepare the mastermix by combining Tubes A, B, C and D and keep in the cool caddy.

⬇

Add ice-cold mastermix to 0.2 mL reaction tubes held in the cool caddy. Mix well and spin down.

⬇

Incubate reactions for 60 minutes at 37°C.

⬇

Place reaction tubes immediately in the cool caddy after incubation. Add ice-cold Detection Buffer (DB) to reaction tubes.

⬇

Incubate reaction tubes for 5 minutes tubes in the cool caddy.

⬇

Measure end-point fluorescence at Ex485 nm / Em520 nm wavelengths using a fluorescence plate reader.

3. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision single pipettes and disposable tips to deliver 0.5 – 1000 µL
- Multi-channel pipette
- Nuclease-free water
- Microplate reader capable of measuring fluorescence at Ex485 nm / Em520 nm
- Microfuge for pulse spins
- Reagent reservoir
- Nuclease free (RNAse-DNAse free) 2 or 5 mL plastic tube
- Incubator set at 37°C or if not available, a heat block set at 37°C
- Cool Caddy (precooled in -20°C)

See the InsituGen website for a list of recommended suppliers for equipment required, and a general set-up guide.

4. MATERIALS SUPPLIED

All kit reagents should be stored at -20°C upon arrival with the exception of the Detection Buffer which should be stored in the dark at 4°C.

Table 1: Specification and quantity of material supplied in the FFX kit

	MATERIALS	SPECIFICATION	QUANTITY
1	96-well black plate	96 wells	1
2	8-strip reaction tubes	0.2 mL	12
3	Tube A	32 reactions/vial	3
4	Tube B	32 reactions/vial	3
5	Tube C	32 reactions/vial	3
6	Tube D	32 reactions/vial	3
7	Equine plasma Control H	>6 reactions/vial	1
8	Equine plasma Control L	>6 reactions/vial	1
9	Detection Buffer (DB)	96 reactions/tube	1

5. SAMPLE COLLECTION AND STORAGE

Plasma: Collect plasma using e.g. heparin as an anticoagulant. Please note that EDTA will interfere with the assay and samples collected in EDTA tubes cannot be used with firstFlagX. Use the fresh plasma immediately or aliquot intro glass vials and store plasma at -80°C.

6. SAMPLE PREPARATION

The kit can be used:

a) In its entirety as 92 tests, or 46 tests in duplicate, using a total of 12 strips. Half of the 1st strip is used for the 2 controls that are i) 'Control High' (H) and ii) 'Control Low' (L). Each control should be performed in duplicate.

b) As one-third, where 28 tests, or 14 tests in duplicate are performed. The 28 tests will use 4 strips. Half of the 1st strip will use the two controls as in a) above. Example layout of assay for b):

Strip 1: 2x Control H, 2 x Control L, 2 or 4 Samples (duplicate or singlet, respectively)

Strips 2 - 4 : 12 or 24 Samples (duplicates or singlet, respectively)

c) Less than one - third of the kit, with minimum 5 tests.
Example layout of assay for c):
Strip 1: 2 x Control H, 2 x Control L, Sample (minimum 5 tests)
Strip 2-4: As required

7. REAGENT PREPARATION

1) The Detection Buffer (DB) is supplied ready to use as 9.6 mL in 1 vial. If the kit is to be divided into three, aliquot the required amount of DB from the bottle. Note, that each reaction requires 80 µL of DB. If aliquoting out for "n" number of samples, multiply 0.08 x { n + 4 Controls] + 10% extra to determine the volume of DB required.
2) Assemble the reactions in the cool caddy or equivalent.
3) Spin down lyophilized tubes A to D for 1 min to ensure all lyophilized contents are at the bottom of the tube. **Reconstitute each lyophilized tubes (A to D) with 128 µL of ice-cold nuclease free water**. Place the tubes in the cool caddy for 5 mins to ensure total reconstitution, followed by a vortexing for ~3 seconds and a spinning down to bring all liquid to the bottom of the tube.
4) Resuspend **Controls H & L tubes in 150 µL ice-cold nuclease-free water**. Leave in cool caddy for 5 minutes, vortex the tubes for ~3 seconds.

8. MASTER MIX PREPARATION

1) **Entire kit (92 samples):** Reconstitute all three sets (sachets labelled “Set 1”, “Set 2” & “Set 3”) each containing lyophilized tubes A, B, C & D with ice-cold nuclease free water as described in Section “Reagent Preparation”. Combine all 3 Tubes A’s into one the A tubes. Repeat for the Tube B’s, C’s and D’s.

   Once reconstituted, assemble the master mix in a nuclease free 2 or 5 mL tube (not provided) held in cool caddy, as below :

   1. 3 X Tube B (384 µL) to Tube A content (384 µL)
   2. 3 X Tube C (384 µL) to Tube A+ B content
   3. 3 X Tube D (384 µL) to Tube A+ B+ C content
      Total Volume : 1536 µL

   Mix thoroughly by pipetting up and down approximately 10 times using a 1000 µL pipette.

2) **One-third of the kit (28 samples):** Reconstitute one set containing lyophilized tubes A, B, C & D with ice-cold nuclease free water as described in “Reagent Preparation” section. After reconstitution, combine the tubes in the following order only:

   1. 1 x Tube A (128 µL) to 1 x Tube B content (128 µL)
   2. 1 x Tube C (128 µL) to Tube A+ B content
   3. 1 x Tube D (128 µL) to Tube A+ B+ C content
      Total Volume : 512 µL

   Mix thoroughly by pipetting up and down approximately 10 times using a 1000 µL pipette.

3) **Less than one-third of the kit:** Reconstitute one set containing lyophilized tubes A, B, C & D with ice-cold nuclease free water as described in “Reagent Preparation”.

   If using a sample number less than 28 samples, calculate the volume of reagent required from each tube, for required samples number “n” + 2 x Control H + 2 x Control L x 16 µL x10% extra.

   The tests can be run with only 1 sample, if required. It would require 5 reactions to allow for the controls to be performed in duplicates. Please use the following table as a guide to calculate the volumes required.

Table 2: Example volumes required from reconstituted FFX tubes for “n” of samples.

TUBE	Volume required from each Tube for 5 reactions (µL)	Volume required for 8 reactions (µL)
Tube A	20	32
Tube B	20	32
Tube C	20	32
Tube D	20	32
Total Volume	80	128

9. ASSAY PROCEDURE

1) Ensure all 0.2 mL reaction tubes are placed in the cool caddy
2) Dispense 16 µL of Master mix to all reaction tubes
3) Add 4 µL of Control H to tubes 1 and 2 (Strip 1)
4) Add 4 µL of Control L to tubes 3 and 4 (Strip 1)
5) Add 4 µL plasma samples to the remaining tubes
6) When adding controls or sample, pipette the samples to the side of the tube, spin down, gently flick with finger, pulse spin the contents to the bottom of the tube. We recommend reverse pipetting- a video showing the method is available on the InsituGen website.
7) Incubate at 37°C for 60 minutes.
8) After incubation, immediately place the tubes in the cool caddy and add 80 µL of detection buffer to each reaction tube and gently mix by pipetting up and down 5 times.
9) Leave the tubes in the cool caddy for 5 minutes.
10) Use a multichannel pipette to gently transfer 80 µL of each reaction to the black flat-bottom 96-well plate. Try not to introduce bubbles. If bubbles form in any of the wells, use a clean pipette tip to burst them.
11) End point read in a fluorescence plate reader at Ex485nm/Em520nm

10. CALCULATION OF RESULTS

1) To calculate the Androgen Receptor (AR) Bioactivity %, use the fluorescent value in the equation below:

$$AR\ Bioacivity\ \% = \frac{Control\ L - Sample}{Conrol\ L}\ x100$$

2) Plotting the AR Bioactivity % versus the testosterone quant value can help identify samples with higher-than- expected bioactivity levels (see the InsituGen website for an example).

11. CERTIFICATE OF COMPLIANCE

1) In the same lot CV%<10%
2) Different lot CV%<10%
3) Spike recovery: 88-94%
4) Sensitivity: The sensitivity of this assay is 4 ng/mL.
5) Specificity: This assay has high specificity for androgen bioactivity. No significant cross-reactivity with estradiol, glucocorticoid or progesterone has been measured.
6) Limited by current skills and knowledge, it is impossible for us to complete cross-reactivity detection between all known progestogens. It does show cross-reactivity with altrenogest and R1881, both of which are known to activate the androgen receptor.

12. SAFETY NOTES AND PRECAUTIONS

1) This kit contains small amounts of dithiothreitol (DTT) and dimethyl sulfoxide (DMSO). Skin contact, eye contact, ingestion and inhalation should be avoided. In case of contact, rinse the affected area with plenty of water. If ingested or inhaled seek medical advice.
2) Plasma should be considered a biologically hazardous material. It is recommended that personal protective equipment is used when working with this kit.
3) This test is based on in vitro transcription technology whereby an RNA molecule is generated from a DNA template. Plasma samples contain endogenous RNases therefore care is needed to ensure that other external sources of RNases are limited. This includes wearing of gloves, not talking or whistling over open tubes, and using nuclease-free water and pipette tips.
4) Biological waste must be disposed of in accordance with federal, state and local environmental control regulations.

13. QUALITY CONTROL

1) Plasma samples should be aliquoted and frozen if not used shortly after collection to avoid repeated freeze-thaw cycles.
2) Prepare reagents on ice and mix before each use.
3) Cover and cap all kit components and store at minus 20°C when not in use. If Tubes A to D, are reconstituted, store as per instructions on Table 3.
4) Do not use reagents after the kit expiration date.
5) Avoid bubbles in 96-well plate.
6) Read fluorescence immediately after transferring to the 96-well black plate. If this is not possible, then keep cold and in the dark until the plate can be read.Cover and cap all kit components and store at -20°C when not in use.

14. STORAGE CONDITIONS

Upon delivery, store the kit at -20°C until use. After opening, the detection buffer should be stored at 4°C in the dark, and the plates and 0.2 mL tubes can be stored at room temperature if preferred.

15. RISK CATEGORY

The risk category according to Regulation 1069/2009/EC, Article 10 is:
Category 3

16. LOT NUMBER AND VALIDITY PERIOD

For information on the LOT Lot number, manufacturing date, and validity period, please refer to the label.

An electronic copy of this instruction manual, instructional resources, and other quality control documentation, are available from the InsituGen website www.insituGen.com

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