Horse 4MID® Kit
User Manual Instructions – R11

PRINCIPLE AND GENERAL DESCRIPTION OF THE HORSE 4MID® KIT (Ref. 4VDX-18K3)

The Horse 4MID® Kit (4VDX-18K3) is a sandwich ELISA assay to detect and quantify proAKAP4 in ejaculate, chilled and frozen semen in extenders or in isolated spermatozoa from stallions. The Horse 4MID® Kit is composed of a 96-well microplate (12 x 8-wells strips), and all the reagents and buffers required to run the assay. The proAKAP4 is a sperm-specific protein that is a marker of sperm quality and male fertility in stallions and main mammals (Blommaert et al. 2018; Ruelle et al. 2019; Sergeant et al. 2019; Delehedde et al. 2019; Blommaert et al. 2019a; Blommaert et al. 2019b; Ruelle et al. 2020; Griffin et al. 2020; Bastan and Akcay, 2021; Blommaert et al. 2021; Boersma et al. 2022; Dordas-Perpinyà et al. 2022). Spermatozoa without proAKAP4 / AKAP4 are abnormal, immotile, and infertile (Miki et al. 2002; Fang et al. 2019; Delehedde et al. 2019). Oxidative stress decreases proAKAP4 concentrations in spermatozoa (Hamada et al. 2013; Nixon et al. 2019; Delehedde et al. 2019). Each 4MID® assay required two steps as described below in Part A and Part B of the Horse 4MID® Kit procedure. The proAKAP4 protein should be first released from spermatozoa flagellum using the Horse Spermatozoa Lysis Buffer and according to the types and/or sperm concentrations of the samples (ejaculate, semen in extenders, or isolated spermatozoa) as described in Part A of the procedure. Then in Part B of the procedure, each prepared semen sample is loaded onto a 96-well microplate where a first antibody coated onto the bottom of the microplate will capture the proAKAP4 protein in the samples and will then be recognized by a second antibody that is covalently coupled to horseradish peroxidase (sandwich method). A Substrate Solution is then added to each well and different color levels (blue) will appear proportionally to the concentrations of the proAKAP4 present in each semen sample. The color reaction is then stopped by adding the Stop Solution and the color intensity (yellow) is measured by spectrophotometry (reading at 450 nm). A positive control is included in each Horse 4MID® Kit. Ready-to-use Standard Solutions are provided to run a Linear Standard Curve allowing the calculation of proAKAP4 concentrations in stallions' semen samples. To compare animals or ejaculates or doses, we always recommend expressing proAKAP4 concentrations in ng per 10 millions spermatozoa (ng/10M spz). Therefore, the concentrations expressed in the number of spermatozoa in the samples should be known before running the assay or be performed independently of the Horse 4MID® assay.

Always read the following instructions carefully before use

TABLE OF CONTENTS

I. REAGENTS AND MATERIALS INCLUDED

R1 – Capture Antibody Coated Microplate of 96-wells (12 x 8-well strips)
R2 - 1 Bottle of 10x Washing Buffer Solution (30 mL)
R3 - 1 Bottle of 1x Dilution Buffer (30 mL)
R4 - 7 Vials of Ready-to-Use Standard Solution (0.7 mL per vial)
R5 - 1 Bottle of 1x Horse Spermatozoa Lysis Buffer (30 mL)
R6 - 1 Vial of Detection Antibody (1 mL)
R7 - 1 Bottle of Substrate Solution (11 mL)
R8 - 1 Bottle 1x Stop Solution (6 mL)
R9 - 1 Vial of Positive Control (0.1 mL)
R10 - 2 Adhesive Plate Sealers
R11 - User manual instructions

II. REAGENT AND MATERIAL REQUIRED - NOT INCLUDED

- 96-well microplate reader measuring absorbance at 450 nm
- Horizontal orbital microplate shaker (300 rpm)
- Vortex
- Multichannel pipette of 300 µL
- Pipettes of 20 µL, 200 µL and 1000 µL
- Pipette tips
- Polypropylene tubes of 1.5 mL (for sample preparation)
- One polypropylene tube of 15 mL (for R6 dilution)
- Plastic reagent reservoirs
- One glass bottle (for R2 dilution)
- Scissors
- Pen
- Aluminum foil
- Ultrapure or double deionized water

III. STORAGE INFORMATION

- The Horse 4MID® Kit should be stored at 4°C upon receipt.
- All reagents must be protected from intense light.

IV. GENERAL INSTRUCTIONS OF USE

- Before use, bring all reagents except the R6 vial **at room temperature (RT) for at least 30 minutes before running the assay**. The R6 Detection Antibody vial should always be kept at 4°C.
- Verify the absence of crystals in the R2 and the R5 bottles. In presence of crystals, gently agitate the solution until all crystals are completely dissolved. Then R2 and R5 solutions should be kept at ambient temperature.
- The R1 96-well Plate (12 x 8-well strips) is in a reusable aluminum foil pouch. The plate frame and unused strips can be placed back in the reusable foil pouch for later use.

A. Preparation of the Semen Samples

ProAKAP4 should be first extracted from spermatozoa flagellum by using a specific R5 Horse Spermatozoa Lysis Buffer.

Do not forget before any pipetting of semen sample (ejaculate, chilled or frozen semen in extenders or isolated spermatozoa) to **resuspend cells by gently shaking** the tube containing the semen as spermatozoa cells will pellet by sedimentation.

Please note that before lysing spermatozoa cells, the semen should always be brought to ambient temperature but **never kept on ice.**

a) Ejaculate or sample with sperm concentration above 50 million of spermatozoa per mL

1. In a 1.5 mL conic tube add 175 µL of R5 Horse Spermatozoa Lysis Buffer.
2. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
3. Add 25 µL of semen to the R5 Horse Spermatozoa Lysis Buffer to reach a volume of 200 µL.
4. Carefully vortex each tube during 1 min at maximum speed **continuously**. All tubes should be vortexed before step 5.

   Remark: Improper vortexing will lead to negative or false quantification value

5. Add 200 µL of R3 Dilution Buffer in the lysed samples.
6. Vortex rapidly at maximum speed.
7. Keep the lysed sample at ambient temperature (17°C – 25°C) not more than 6 hours before use. Do not put on ice.

b) Semen in extenders (chilled or frozen) or any sample with sperm concentration comprised between 10 to 50 million of spermatozoa per mL

1. In a 1.5 mL conic tube add 160 µL of R5 Horse Spermatozoa Lysis Buffer.
2. Unfroze the semen samples following the user proper procedure.
3. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
4. Add 40 µL of Semen to the R5 Horse Spermatozoa Lysis Buffer to reach a volume of 200 µL.
5. Carefully vortex each tube during 1 min at maximum speed **continuously**. All tubes should be vortexed before step 6.

   Remark: Improper vortexing will lead to negative or false quantification value

6. Add 200 µL of R3 Dilution Buffer in the lysed samples.
7. Vortex rapidly at maximum speed.
8. Keep the lysed samples at ambient temperature (17°C – 25°C), not more than 6 hours before use. Do not put on ice.

c) Isolated spermatozoa

1. Resuspend the spermatozoa pellet containing 1 000 000 spermatozoa with 150 µL of the R5 Horse Semen Lysis Buffer.

2. Carefully vortex each tube for 1 min at maximum speed **continuously**. All tubes should be vortexed before step 3.

   Remark: Improper vortexing will lead to negative or false quantification value

3. Add 200 µL of the R3 dilution buffer to each sample.
4. Vortex rapidly at maximum speed
5. Keep the lysed samples at ambient temperature (17°C – 25°C) not more than 6 hours before use. Do not put it on ice.

B. Dosage of proAKAP4 Concentrations Using the Horse 4MID® Kit

1. Open carefully the reusable aluminum foil pouch containing the R1 Microplate using scissors

2. Add 100 µL of each Standard Solution (vial n°R4-1 to vial n° R4-7) on the first 8-well strip **from A1 well to G1 well** of the R1 Microplate (see table below). Please note that the R4-7 Standard Solution is the **Negative Control**. Always use a new tip for each different solution.

Standard vial n°	R4-1	R4-2	R4-3	R4-4	R4-5	R4-6	R4-7
ng / mL of proAKAP4	150	75	37.5	18.75	9.4	4.7	0
Well Position	A1	B1	C1	D1	E1	F1	G1

Always follow this microplate scheme if using the calculation datasheet provided by 4BioDx® services.

3. Add 100 µL of the **R9 Positive Control** in H1 well on the first strip of the R1 Microplate.

4. Then add **150 µL of each semen sample as prepared in Part A**. Up to 88 samples can be analyzed on the R1 Microplate from **well A2 to well H12** (from strip 2 to strip 12).

   Remark 1: Vortex rapidly each semen sample before loading
   Remark 2: Always use a new tip for each different semen sample.

5. Cover the R1 Microplate with one R10 Adhesive Plate Sealer and incubate for 2 hours at room temperature on a horizontal shaker with gentle agitation (200 rpm).

6. During the incubation time:

   a. Prepare the Detection Antibody: Dilute the content of the R6 Detection Antibody tube in 11 mL of R3 Dilution Buffer in a 15 mL tube. Close the tube and mix the Detection Antibody Solution by reversing the 15 mL tube several times. Keep this solution at ambient temperature until Step 9.

      Remark 1: Before dilution in R3, please carefully hit the bottom of the R6 tube vertically or rapidly centrifuge to pull down any liquid in the cap of the R6 tube.

      Remark 2: After dilution in R3, the Detection Antibody Solution should be used within one month and kept at 4°C.

   b. Prepare the Washing Solution: Add 30 mL of the R2 into 270 mL of ultrapure water (or double deionized water) in a clean glass bottle. Gently agitate the solution, avoid foaming and keep it at ambient temperature.

7. At the end of incubation time, remove the R10 Adhesive Plate Sealer and eliminate the sample solutions by R1 Microplate reversion (or aspirate when using an automatic microplate washer).

8. Transfer some Washing Buffer to a plastic reagent reservoir. Wash each well by adding **300 µL of R2 Washing Buffer 1x Solution** using the multichannel micropipette. Then discard the Washing Solution by reversion of the R1 Microplate. Repeat two times more. Please tap down gently on the R1 Microplate on an absorbent dry paper to remove residual R2 Washing Buffer droplets between each washing step and before loading the R6 Detection Antibody.

   Remark: Results of the assay will markedly be influenced by the proper performance of washing steps.

9. Transfer all the Detection Antibody Solution as prepared in step 6 into a plastic reagent reservoir. **Add 100 µL of Detection Antibody Solution** to each well of the 96-well R1 Microplate using the multichannel micropipette.

10. Cover the plate with a new R10 Adhesive Plate Sealer and incubate the R1 Microplate **for one hour** at room temperature with gentle agitation (300 rpm).

11. Remove the R10 Adhesive Plate Sealer and eliminate the Antibody Solution by R1 Microplate reversion (or aspiration when using an automatic microplate washer).

12. Transfer some Washing Buffer to a plastic reagent reservoir. Wash each well by adding **300 µL of R2 Washing Buffer 1x Solution** using the multichannel micropipette (or an automatic microplate washer). Then discard the Washing Solution. Repeat two times more. Please tap down gently on the R1 Microplate on an absorbent dry paper to remove the residual Washing Buffer droplet between each washing step and before adding the R7 Substrate.

    Remark: Results of the assay will markedly be influenced by the proper performance of the washing steps.

13. Transfer the Substrate Solution to a plastic reagent reservoir. Add **100 µL of R7 Substrate Solution** to each well using the multichannel pipette.

    Remark 1: Keep carefully away from light with an aluminum sheet.

    Remark 2: Please note that R7 Substrate Solution and R8 Stop Solution (step 15) should be added to the wells in the same order and with the same time interval.

14. Protect from light and incubate the R1 Microplate under gentle agitation (300 rpm) for **10 minutes** at room temperature.

    Remark: The incubation must not exceed 30 minutes.

15. Transfer the Stop Solution to a plastic reagent reservoir. Add **50 µL of R8 Stop Solution** to each well using the multichannel pipette and place the R1 Microplate for 2 minutes on an orbital shaker at 300 rpm to mix well before reading the R1 Microplate.

16. Determine the optical density using a microplate reader set to 450 nm.

    *Remark: Always perform the measure **immediately** after adding the Stop Solution.*

C. Calculation of Results – ProAKAP4 Concentration Determination

A Standard Curve must be performed for each assay to calculate proAKAP4 concentrations in bull semen samples. You can either do the Standard Curve a) using the Calculation Data Sheet provide for each sperm preparation protocol (part A) or b) do it manually yourself using software such as Excel.

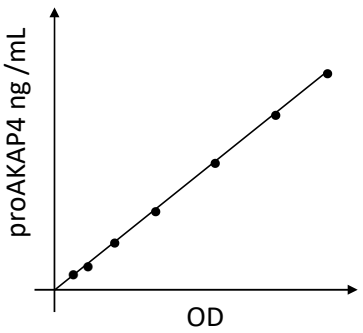
Remark: always consider the dilutions you have made when considering the sperm concentration of the semen samples.

a) Using the Calculation Data Sheets provide by 4BioDx® services (upon request at contact@4biodx.com). The Calculation Data Sheets have been designed to automatically draw the Standard Curve from your optical density values (OD) and calculate the proAKAP4 concentrations first in ng /mL, then in ng/10 million of spermatozoa. First select the calculation data sheet according to the sample preparation procedure (a, b or c) you have used in Part A. You will need to fill the blue cases with OD obtained from the plate reader and follow the indicated steps on the sheet to draw the curve and then the sheet will automatically calculate the concentrations of proAKAP4 in ng/mL. By adding the concentration of spermatozoa in million per mL of the semen samples, or the number of spermatozoa in the case of isolated spermatozoa (blue column of step 5), you will obtain the concentrations of proAKAP4 in ng / 10 million of spermatozoa. You may then copy and paste the values, tables, and graphs into your report files. Please note that hitting the step 6 button will generate a new sheet with the summary of the results.

b) Manually, you will need to first subtract the optical density obtained from value G1 to the optical density of each standard (A1 to G1) and each sample (A8 to H12) that you obtained from the plate reader. Then create a Standard Curve by reporting the data (optical density values) on an Excel Spreadsheet.

Express the optical densities (OD) in abscissa in the function of the proAKAP4 quantities in ng/mL in ordinate and then generate a two-degree polynomial regression equation (ProAKAP4 [conc] = an x $(OD)^2$ + b x (OD)) using the data analysis tools of an Excel Spreadsheet.

ProAKAP4 in (ng / mL)	Optical density (OD) values
150	OD A1
75	OD B1
37.5	OD C1
18.75	OD D1
9.4	OD E1
4.7	OD F1
0	OD G1



Then to **express the proAKAP4 concentrations in ng/mL**, calculate the concentrations using this two-degree polynomial regression equation.

To compare animals or ejaculates or doses, we recommend expressing proAKAP4 concentrations **in ng per 10 million spermatozoa (ng/ 10 M of spz).**

To express results in ng of proAKAP4 per 10 million spermatozoa, the formula will be:
Conc. proAKAP4 ng/ 10 M = (conc. proAKAP4 in ng/mL / conc. spz M/mL) x 10 x dilution factor* x (2/3)
(*use the dilution factor of 20 and 16 for the sample preparation procedure a) or b), respectively.

In the case of isolated spermatozoa: you should first calculate the concentration of spermatozoa in Million per mL (M/mL). To do so, multiply by 2.5 the number of spermatozoa in the pellet that normally contains 700 000 spermatozoa at step 2a of the Semen sample preparation part.

Express then the results in ng of proAKAP4 per 10 million spermatozoa (ng/10M of spz) with the following formula: Conc. proAKAP4 in ng/ 10 M of spz = (conc. proAKAP4 in ng/mL / conc. spz M/mL) x 10 x 6.66

Example of threshold values of proAKAP4 concentrations in stallion semen (all breeds):

ProAKAP4 concentrations	Semen Quality	Long Lasting Motility
Less than 15 ng/10M of spz	Poor	+/-
Between 15 and 40 ng/10M of spz	Good	+
Between 40 and 60 ng/10M of spz	Very Good	++
Over 60 ng/10M of spz	Excellent	+++

V. PRACTICAL ADVICE AND CAUTIONS

- For research use only purposes and not for use for diagnostic purposes.
- Always follow good laboratory practices.
- Always carefully read the instructions before use.
- Always follow the plating scheme indicated in part B when using the Calculation Datasheet provided by 4BioDx® services.
- A Standard Curve should be performed at each assay.
- To avoid distortions due to differences in incubation times, R7 Substrate Solution and R8 Stop Solution should be added to wells in the same order and with the same time interval.
- The use of a multichannel pipette is mandatory to ensure the timely delivery of liquids.
- Use the supplied reagents as an integral unit before the expiration date.
- The R7 Substrate Solution can be irritating to the skin.
- The R8 Stop Solution can be harmful in case of ingestion and could lead to irritation when in contact with the skin.
- Results of the assay will markedly be influenced by the proper performance of the washing steps.
- Do not expose the R7 Substrate Solution to light or oxidative substances.
- Use only reagents from the same Horse 4MID® Kit.
- After dilution in R3, the Detection Antibody Solution should be used within one month and always kept at 4°C.
- Any variation in ambient temperature, pipetting, washing method, or incubation time can cause variation in optical density results.
- Always use a new tip for each different solution and for each sample.
- Observe all federal, state, and local regulations in terms of waste disposal.

VI. REFERENCES

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Check out www.4biodx-breeding.com website or contact us for an updated list of scientific references

VII. RELATED PRODUCTS

Reference	Designation	Specificity
4VDX-18K2	Pig 4MID® Kit	Pig proAKAP4
4VDX-18K3 / 4VDX-18K3BB	Horse 4MID® Kit	Horse / Donkey proAKAP4
4VDX-18K4	Bull 4MID® Kit	Bull proAKAP4
4VDX-18K5 / 4VDX-18K5BB	Dog 4MID® Kit	Dog proAKAP4
4VDX-18K6	Rabbit 4MID® Kit	Rabbit proAKAP4
4VDX-18K7	Ram 4MID® Kit	Ram proAKAP4
4BDX-18K8 / 4BDX-18K8BB	Mouse 4MID® Kit	Mouse / Rat proAKAP4
4VDX-18K9	Goat 4MID® Kit	Caprine proAKAP4
4VDX-19K10	Cat 4MID® Kit	Cat proAKAP4 (feline)
4VDX-19K11	Camel 4MID® Kit	Camelids proAKAP4

FOR RESEARCH USE ONLY PRODUCT
NOT FOR USE IN DIAGNOSTIC PROCEDURES