Goat 4MID® Kit
User Manual Instructions – R11

PRINCIPLE AND GENERAL DESCRIPTION OF THE GOAT 4MID® KIT (Ref. 4VDX-18K9)

The Goat 4MID® Kit (4VDX-18K9) is a sandwich ELISA assay that aims to detect and quantify proAKAP4 in spermatozoa from bucks of all main goat breeds (ejaculate, chilled or frozen semen in extender or in isolated spermatozoa). The Goat 4MID® Kit is composed of a 96-well microplate (12 x 8-well strips), and all the reagents and buffers required to run the assay. The proAKAP4 is a sperm-specific protein that is marker of sperm quality and male fertility in main mammals (Sergeant et al. 2019; Delehedde et al. 2019; Ruelle et al. 2019; Carracedo et al. 2020; Ruelle et al. 2020; Griffin et al. 2020; Bastan and Akcay, 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 (Nixon et al. 2019; Delehedde et al. 2019). Each 4MID® assay requires two major steps as described below at Part A and Part B of the Goat 4MID® Kit procedure. The proAKAP4 protein should be first released from spermatozoa using the Goat 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 Goat 4MID® procedure. Then at 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 contained in the samples and will then be identified using 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 Goat 4MID® Kit. Ready-to-use Standard Solutions are provided to run a Linear Standard Curve allowing the calculation of proAKAP4 concentrations in buck semen samples. To compare animals or ejaculates or doses, we always recommend to express proAKAP4 concentrations in ng per 10 million of spermatozoa (ng/ 10 M of spz). Therefore, the concentrations in number of spermatozoa of the samples should be known before running the assay or be performed independently of the Goat 4MID® assay.

Always read the following instructions carefully before use

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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 Bull Spermatozoa Lysis Buffer (30 mL)
R6 - 1 Vial of Detection Antibody (0.1 mL)
R7 - 1 Bottle of Substrate Solution (11 mL)
R8 - 1 Bottle 1x Stop Solution (6 mL)
R9 - 1 Vial of Positive Control (0.7 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 microtubes of 1.5 mL (for sample preparation)
- One glass bottle (for R2 dilution)
- One polypropylene tube of 15 mL (for R6 dilution)
- Plastic reagent reservoirs
- Scissors, pen, aluminum foil
- Ultrapure or double deionized water

III. STORAGE INFORMATION

- The Goat 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 Goat 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 Goat 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 Goat Spermatozoa Lysis Buffer to reach a volume of 200 µL.
4. Carefully vortex each tube during 1 min at maximum speed **continuously** (3000 rpm or above). 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 each sample.
6. Vortex rapidly at maximum speed.
7. Keep the lysed samples at ambient temperature (17°C – 25°C) not more than 6 hours before use. Do not put on ice.

b) *Semen in extender or 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 Goat 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 Goat 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 each sample.
7. Vortex 1 min at maximum speed.
8. Keep the lyzed 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 and add 140 µL of the R5 Goat Lysis Buffer.
2. Carefully vortex each tube during 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 150 µL of the R3 dilution buffer in 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 on ice.

B. Dosage of ProAKAP4 Concentrations Using the Goat 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 plate scheme if using the calculation datasheet provided by 4BioDx® services.

3. Add **100 µL of the R9 Positive Control** in H1 well of 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 the R1 Microplate for **2 hours** at room temperature on a horizontal shaker with gentle shaking (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 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 at ambient temperature.

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

8. Transfer some Washing Buffer in 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 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 the washing steps.

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

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

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

12. Transfer some Washing Buffer in 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. Repeat two times more. Please tap down gently the R1 Microplate on an absorbent dry paper to remove 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 in a plastic reagent reservoir. Add **100 µL of R7 Substrate Solution** to each well using the multichannel pipette.

    Remark1: 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 wells in the same order and with the same time interval.

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

    Remark: The incubation must not exceed 30 minutes.

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

16. Determine the optical density of all the samples 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 analysis to calculate proAKAP4 concentrations in buck semen. You can either do the Standard Curve a) using the Calculation Data Sheets provided for each sperm preparation protocol (part A) or b) do it manually yourself using software such as Excel.

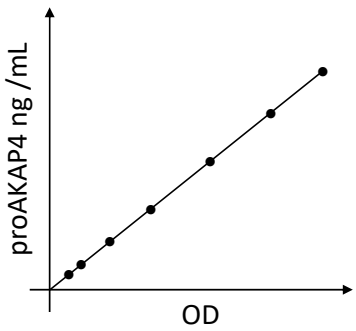
Remark: always take in account the dilutions you have made when considering sperm concentration of the semen samples.

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 then to fill the blue cases with OD obtained from the plate reader and to 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 the 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 on your own report files. Please note that hitting the step 6 button will generate a new sheet with 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] = a 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 to express proAKAP4 concentrations **in ng per 10 million of spermatozoa (ng/ 10 M of spz).**

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

Examples of threshold values of proAKAP4 concentrations in goat 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 follow the plate scheme indicated in part B when using the Calculation Datasheet provided by 4BioDx® services.
- After dilution in R3, the Detection Antibody Solution should be used within one month and always kept at 4°C.
- The use of a multichannel pipette is mandatory to ensure the timely delivery of liquids.
- Use the supplied reagents as an integral unit prior to the expiration date.
- The R7 Substrate Solution can be irritating for the skin.
- The R8 Stop Solution can be harmful in case of ingestion and could lead to irritation when in contact with the skin.
- 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.
- Do not expose the R7 Substrate Solution to light or oxidative substances.
- Use only reagents from the same Goat 4MID® Kit.
- Any variation in ambient temperature, pipetting, washing method or incubation time can cause variation in optical density results.
- Always use 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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Complete list of publications upon request at: contact@4biodx-breeding.com

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/ 4BDX-19K10BB	Cat 4MID® Kit	Cat proAKAP4 (feline)

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4VDX-19K11	Camel 4MID® Kit	Camelids proAKAP4

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