Ram 4MID® Kit
User Manual Instructions

PRINCIPLE AND GENERAL DESCRIPTION OF THE RAM 4MID® KIT (Ref. 4VDX-18K7)

The Ram 4MID® Kit (4VDX-18K7) is a quantitative sandwich ELISA assay to detect and quantify proAKAP4 in ovine sperm samples (fresh or frozen samples). The Ram 4MID® Kit is composed of a 96-well plate and all necessary reagents to run the assay. Protocols for sperm sample preparations are provided to be used with the Ram Spermatozoa Lysis Buffer before loading on the 96-well plate of the Ram 4MID® Kit. The proAKAP4 is recognized by the Capture Antibody coated onto the bottom of the 96-well plate and then detected using a Detection Antibody covalently coupled to horseradish peroxidase. A Substrate Solution is added to each well and color levels appear proportionally to the concentration of the proAKAP4 present in each sperm sample. The color reaction is stopped by the Stop Solution and the color intensity is measured by spectrophotometry at 450 nm. A positive control is provided in each Ram 4MID® Kit. Ready-to-use Standard Solutions are provided to run a Linear Standard Curve and to calculate the proAKAP4 concentrations in ovine semen samples.

Always carefully read the following instructions before use

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I. REAGENTS AND MATERIALS INCLUDED

R1 - 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 Ram Spermatozoa Lysis Buffer (30 mL)
R6 - 1 Vial of Detection Antibody (0.1 mL)
R7 - 1 Bottle of Substrate Solution (12 mL)
R8 - 1 Bottle 1x Stop Solution (6 mL)
R9 - 1 Vial of Positive Control (0.7 mL)
R10 - 2 Adhesive Plate Sealers
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II. MATERIAL REQUIRED - NOT INCLUDED

Microplate reader measuring absorbance at 450 nm
Horizontal orbital microplate shaker
Vortex
Multichannel pipette of 300 µL or multichannel pipette of 1200 µL
Pipettes of 20 µL, 200 µL and 1000 µL
Pipette tips
Polypropylene tubes (1.5 mL and 15 mL)
Plastic Reagent reservoirs
Fridge and freezer
Scissors
Pen
Aluminum foil
Ultrapure or double deionized water

III. STORAGE INFORMATION

- The Ram 4MID® Kit should be stored at 4°C at reception.
- All reagents must be protected from intense light.
- 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 in a reusable foil pouch for later use.
- All remaining reagents need to be stored at 2°C - 8°C until the expiration date indicated.

IV. GENERAL INSTRUCTIONS OF USE

- Before use, bring all reagents except the R6 vial at room temperature (RT) for at least 30 minutes. 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 the presence of crystals, gently agitate the solution until all crystals are completely dissolved.
- R2 and R5 solutions should be kept at ambient temperature.

A. Preparation of the Washing Solution

Always prepare the Washing Solution in a clean glass bottle and use ultrapure or double deionized water.

Prepare the 1x Washing Buffer by a 10-fold dilution of the 10x concentrated R2 Washing Buffer Solution in ultrapure or double deionized water:

- Add 30 mL of the R2 into 270 mL of ultrapure water.
- Gently agitate the solution and avoid foaming.

B. Preparation of the Semen Samples

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

Do not forget before any pipetting of semen sample (fresh raw ejaculate or frozen semen in straws) to resuspend cells by gently shaking the tube containing the semen as spermatozoa cells will pellet by gravity.

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

a) Fresh Ejaculate

1. In a 1.5 mL conic tube add 90 µL of R5 Ram Spermatozoa Lysis Buffer.
2. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
3. Add 30 µL of semen to the R5 Ram Spermatozoa Lysis Buffer to reach a volume of 120 µL.
4. Carefully Vortex continuoulsy during 1 min at maximum speed.
   Remark: Improper vortexing will lead to negative or false quantification value
5. Add 120 µL of R3 Dilution Buffer.
6. Vortex rapidly at maximum speed.
7. Keep at ambient temperature (17°C – 25°C) until use. Do not put on ice.

Remark: For storage longer than 4 hours, please store at -20°C (for up to one week).

b) Frozen Semen

1. In a 1.5 mL conic tube add 80 µL of R5 Ram Spermatozoa Lysis Buffer.
2. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
3. Add 40 µL of Semen to the R5 Ram Spermatozoa Lysis Buffer to reach a volume of 120 µL.
4. Carefully Vortex precisely during 1 min at maximum speed.
5. Add 120 µL of R3 Dilution Buffer.
6. Vortex 1 min at maximum speed.
7. Keep at ambient temperature (17°C – 25°C) until use. Do not put on ice.
   Remark: For storage longer than 4 hours, please store at -20°C (for up to one week).

c) Isolated Spermatozoa

1. Resuspend the spermatozoa pellet with the R5 Ram Lysis Buffer to reach a final concentration of 300 millions of spermatozoa / mL.
2. Carefully vortex continuously during 1 min at maximum speed.

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

3. Pipette 150 µL of the lysate and add 100 µL of the R3 dilution buffer.
4. Vortex rapidly at maximum speed
5. Keep the lysed sample at ambient temperature (17°C – 25°C), not more than 4 hours before use. Do not put on ice

   Remark: For storage longer than 4 hours, please store at -20°C (for up to one week).

C. Dosage of proAKAP4 Using the Ram 4MID® Kit

1. Open carefully the reusable aluminum foil pouch containing the R1 96-well Plate using scissors.
2. Add 100 µL from the highest to the lowest concentrations of the Standard Solution (tube n°R4-1 to tube n° R4-7) on the first 8-well strip **(from A1 to G1)** of the R1 96-well Plate to establish the Standard Curve. Please note that the **R4-7 Standard Solution is the Negative Control**.

Standard tube n°	R4-1	R4-2	R4-3	R4-4	R4-5	R4-6	R4-7
ng / mL of proAKAP4	300	150	75	37.5	18.75	9.4	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. Then add 100 µL of the **R9 Positive Control** in H1 well
4. Analyze up to 88 samples by adding 150 µL of each semen sample as prepared above **(from A2 to H12).**
5. Cover the R1 plate with one R10 Adhesive Plate Sealer and incubate for the indicated time in the table below at room temperature on a horizontal shaker with gentle agitation (300 rpm).

Type of sample	Incubation time
Fresh ejaculate	1h30
Straw or Isolated spermatozoa	2h00

6. During the incubation time, dilute the 60 µL of R6 Detection Antibody in 12 mL of R3 Dilution Buffer in a 15 mL tube.
7. Remove the R10 Adhesive Plate Sealer and eliminate the samples by plate reversion (or aspirate when using an automatic microplate washer).
8. 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 the 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.

9. Add 100 µL of Detection Antibody solution prepared in step 6 to each well of the 96-well Plate (R1) using the multichannel micropipette.

10. Cover the plate with a new R10 Adhesive Plate Sealer and incubate for the indicated time in the table below at room temperature with gentle agitation (300 rpm).

Type of sample	Incubation time
Fresh ejaculate	30 minutes
Straw or Isolated spermatozoa	1 hour

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

12. 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 the microplate on an absorbent dry paper to remove residual Washing Buffer droplet between each washing step and before adding the R7 Substrate.

13. Add 100 µL of R7 Substrate Solution to each well using the multichannel pipette.
Remark: Keep carefully away from light with an aluminium sheet.

14. Protect from light and incubate under gentle agitation (300 rpm) for 10 minutes at RT.
Remark: The incubation must not exceed 30 minutes.

15. Add 50 µL of R8 Stop Solution to each well using the multichannel pipette and mix 2 minutes at 300 rpm on an orbital shaker before reading the plate.

16. Determine the optical density of the samples using a microplate reader set to 450 nm.
*Remark: Always perform the measure **immediately** after adding the Stop Solution.*

D. Calculation of Results – ProAKAP4 Concentration Determination

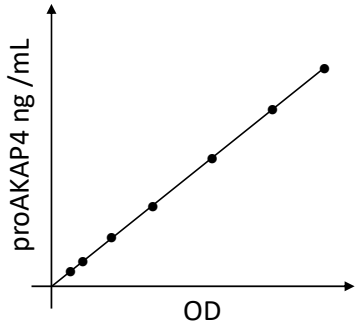
A Standard Curve must be performed for each analysis to calculate proAKAP4 concentrations in ram semen samples.

You can either do it manually yourself using software such as Excel or using the calculation sheet provide upon request by 4BioDx® services (at contact@4biodx.com).

Manually, you will need to subtract the optical density obtained from value G1 to the optical density of each standard (A1 to G1) and each sample (A2 to H12). Then create a Standard Curve by reporting the data (optical density values) on an Excel Spreadsheet.

Express the optical densities (OD) in abscissa in 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 the Excel Spreadsheet.

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



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

To compare animals or ejaculates or doses, we recommend to express proAKAP4 concentrations in ng per 10 millions of spermatozoa (ng / 10 M).

To express results in ng of proAKAP4 per 10 millions 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 indicated above either for ejaculate or for dose)

Automatically, you may want to use the Calculation Data Sheet provide by 4BioDx® services (upon request at contact@4biodx.com). You will only need to follow the indicated steps to draw the curve and automatically calculate the concentrations of proAKAP4 in ng / mL and in ng / 10 millions of spermatozoa. And easily obtain the tables and graphs of your results that you can copy and paste on your own report files.

V. Practical Advices and Cautions

- For Research Use Only and not for use for diagnostic purposes.
- A Standard Curve should be performed at each assay.
- When performing the assay, standards and samples should be pipetted in less than 15 minutes.
- Always follow the plate scheme indicated in part C, if using the calculation datasheet provided by 4BioDx® services.
- 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 recommended to ensure the timely delivery of liquids and washing steps.
- Always follow good laboratory practices.
- Use the supplied reagents as an integral unit prior to the expiration date.
- The Substrate Solution can be irritating for the skin.
- Do not expose the Substrate Solution to light or oxidative substances.
- The Stop Solution can be harmful in case of ingestion and could lead to irritation when in contact with the skin.
- Use only reagents from the same Ram 4MID® Kit.
- Any variation in ambient temperature, pipetting, washing method or incubation time can cause variation in optical density results.
- Observe all federal, state, and local regulations in terms of waste disposal.

REFERENCES

Riesco et al. (2020) Biomolecules. 10(7):E1046
Xu et al. (2020) Development. 147(2)
Sergeant et al. (2019) Dairy Vet. Sci. J. 11(1): 803-811
Dewulf et al. (2019) Dairy Vet. Sci. J. Vol. 13(3):1-7
Nixon et al. (2019) Front Cell Dev Biol Vol. 7(319): 1-18
Blommaert et al. (2019) Theriogenology. 131: 52-60
Singh et al. (2019) Theriogenology. Vol. 129: 130-145
Fu et al. (2019) Theriogenology. Vol. 134: 74-82
Fang et al. (2019) Developmental Biology, In Press
Delehedde et al. (2018) Animal Reproduction Science. Vol.194: 24
Sergeant et al. (2016) Animal Reproduction Science. Vol.169: 125-126
Peddinti et al. (2008) BMC Systems Biology. Vol.2: 19
Miki et al. (2002) Developmental Biology. Vol. 248: 331-342

RELATED PRODUCTS

Reference	Designation	Specificity
4VDX-18K2	Pig 4MID® Kit	Pig proAKAP4
4VDX-18K3	Horse 4MID® Kit	Horse proAKAP4
4VDX-18K4	Bull 4MID® Kit	Bovine proAKAP4
4VDX-18K5	Dog 4MID® Kit	Dog proAKAP4
4VDX-18K6	Rabbit 4MID® Kit	Rabbit proAKAP4
4VDX-18K7	Ram 4MID® Kit	Ram proAKAP4
4VDX-18K8	Mouse 4MID® Kit	Mouse / Rat proAKAP4
4VDX-18K9	Goat 4MID® Kit	Caprine proAKAP4
4VDX-19K10	Cat 4MID® Kit	Cat proAKAP4
4VDX-19K11	Camel 4MID® Kit	Camel / Alpaga proAKAP4

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