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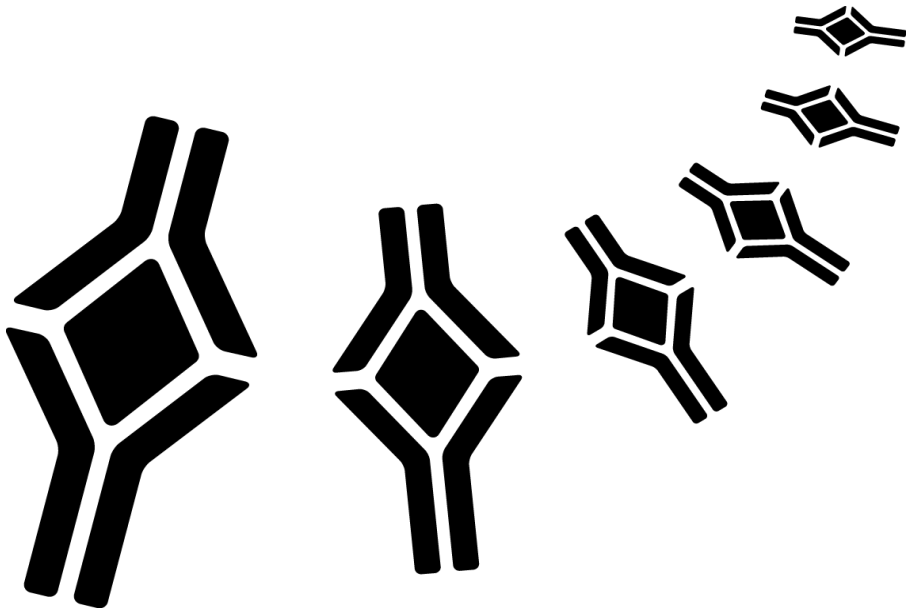


HUMAN UNACYLATED GHRELIN ELISA

Product Data Sheet

Cat. No.: RA194063400R

For Research Use Only

CONTENTS

» **This kit is manufactured by:**
BioVendor – Laboratorní medicína a.s.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1 INTENDED USE

96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

- A covered 96 well plate, pre-coated with anti-Ghrelin mouse monoclonal antibody, ready to use
- One vial of Conjugate Solution, lyophilised
- Two vials of Human unacylated ghrelin standard, lyophilised
- Two vials of Quality Control, lyophilised
- One vial of Dilution Buffer, lyophilised
- One vial of Wash Solution Concentrate, liquid
- One vial of Tween 20, liquid
- Two vials of Substrate Solution (Ellman's reagent), lyophilised
- One Product Data Sheet
- One Certificate of Analysis
- One well cover sheet

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 32 samples in duplicate.

2 STORAGE, EXPIRATION

Store the complete kit at -20°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

3 PRECAUTIONS FOR USE

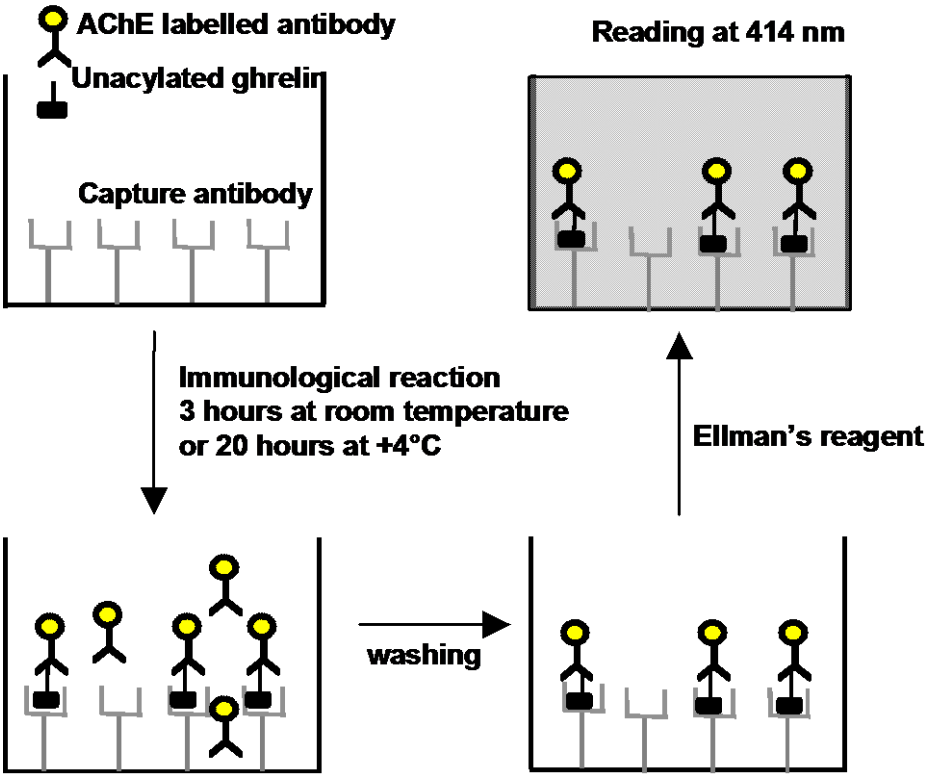
Users are recommended to read all instructions for use before starting work.
Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation two or three times before distribution.

For research laboratory use only.
Not for diagnostic use.
Do not pipet liquids by mouth.
Do not use kit components beyond the expiration date.
Do not eat, drink or smoke in area in which kit reagents are handled.
Avoid splashing.
The total amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

4 PRINCIPLE OF THE ASSAY

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized principally in the stomach. It stimulates food intake and transduces signals to hypothalamic regulatory nucleid that controls energy homeostasis. The peptide consists of 28 amino acids, with a n-octanoylation of the serine-3 residue, which is necessary for the biological activity mentioned below. Ghrelin is present in the peripheral circulation under two forms: acylated and non-acylated. The human unacylated ghrelin EIA kit specifically measures the unacylated form of ghrelin. This Enzyme Immunometric Assay (EIA) is based on a double-antibody sandwich technique. The wells of the plate supplied with the kit are coated with a monoclonal antibody specific to the C-terminal part of ghrelin. This antibody will bind to any ghrelin introduced into the wells (standard or sample). The acetylcholinesterase (AChE) - Fab' conjugate which recognises the N-terminal part of unacylated ghrelin is also added to the wells. This allows the two antibodies to form a sandwich by binding on different parts of the human unacylated ghrelin.

The sandwich is immobilised on the plate so the excess reagents may be washed away. The concentration of the human unacylated ghrelin is then determined by measuring the enzymatic activity of the immobilized AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound. The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of the human acylated ghrelin present in the well during the immunological incubation.



The principle of the assay is summarised below:

5 MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

5.1 FOR SAMPLE PREPARATION

+ Ethylenediaminetetra-acetic acid (EDTA)
+ Potassium Phosphate buffer 0.1 M pH 7.4

5.2 FOR THE ASSAY

+ Precision micropipettes (20 to 1000 µL)
+ Spectrophotometer plate reader (405 or 414 nm filter)
+ Microplate washer (or wash-bottles)
+ Microplate shaker
+ Distilled or deionized water
+ Polypropylene tubes

6 SAMPLE PREPARATION

6.1 GENERAL PRECAUTIONS

+ All samples must be free of organic solvents prior to assay.
+ Samples should be assayed immediately after collection or should be stored at -20°C.

6.2 BLOOD COLLECTION

Blood samples are collected in tubes containing EDTA. Samples are centrifuged at 3,500 rpm for 10 minutes at +4°C and then, supernatants are transferred in separate tubes. Samples should be quickly assayed or stored at -20°C for later use.
For assaying the acylated ghrelin, please refer to the section “Blood collection” of the protocol of the RA194062400R Human Acylated Ghrelin Kit .

6.3 SAMPLE PREPARATION

Plasma samples may be directly assayed (without any extraction procedure) after being diluted at least to 1:10 in Dilution buffer in order to avoid matrix effect.

7 REAGENT PREPARATION

The coated plates and reagents are provided ready to use.

7.1 7.1 Dilution Buffer

Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

7.2 Human unacylated ghrelin standard

Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 250 pg/mL. Prepare seven propylene tubes and add 500 µL of Dilution Buffer into each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 250 (S1), 125 (S2), 62.5 (S3), 31.3 (S4), 15.6 (S5), 7.81 (S6), 3.91 (S7) and 1.96 pg/mL (S8), respectively. Stability at -20°C: 1 week.

7.3 Quality Control

Reconstitute the vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at -20°C: 1 week.

7.4 Conjugate Solution

Reconstitute one vial with 10 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 week.

7.5 Wash Solution Concentrate

Dilute 1 mL of Wash Solution Concentrate to 400 mL with distilled or deionized water. Add 200 µL of tween 20 (use a magnetic stirrer to mix the contents). Stability at 4°C: 1 week.

7.6 Substrate Solution

Five minutes before use, reconstitute with 49 mL of distilled or deionized water and 1 mL of concentrated Wash Solution Concentrate. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark: 1 day. It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

8 ASSAY PROCEDURE

8.1 PLATE PREPARATION

Prepare the Wash Solution Concetrate as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4°C). Rinse each well 5 times with the Wash Solution Concentrate (300 µL/well).

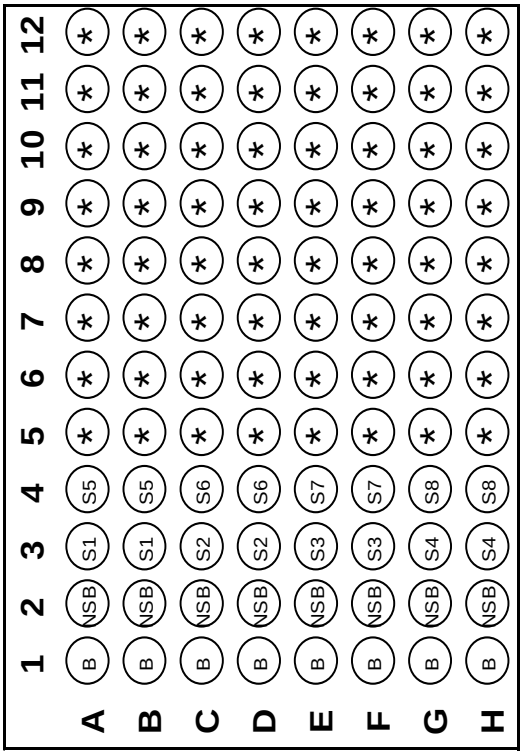
Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and dry by inversion on absorbent paper.

8.2 DISTRIBUTION OF REAGENTS AND SAMPLES

A plate set-up is suggested on this page. The contents of each well may be recorded on the sheet provided with the kit.

8.3 PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Ellman's reagent. All samples and reagents must reach room temperature prior performing the assay. Use different tips to pipet the buffer, standard, quality control, samples, tracer, and other reagent



NSB : Non Specific Binding

S1-S8: Standards 1-8

* : Samples or Quality Controls

- Dilution Buffer:

Dispense 100 µL to Non Specific Binding (NSB) wells.

- Human unacylated ghrelin standard:

Dispense 100 µL of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

- Quality Control :

Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

- Conjugate Solution:

Dispense 100 µL to each well, except Blank (B) wells.

8.4 INCUBATING THE PLATE

Cover the plate with adhesive film and incubate for: 3 hours at room temperature, or - 20 hours at +4°C. The long immunological reaction allows the increase of assay sensitivity: 0.2 pg/mL versus 0.6 pg/mL for short immunological reaction.

8.5 DEVELOPING AND READING THE PLATE

Reconstitute Ellman's Reagent as indicated in reagent preparation section. Wash each well five times with the wash buffer (300 µL/well), slightly shake the plate for 5 minutes (with the orbital shaker) and then rewash 5 times with the wash buffer (300 µL/well). Remove the liquid from the wells by inverting the plate. Dry by inversion on absorbent paper.

Dispense 200 µL of Ellman's Reagent to the 96 wells. Incubate the plate in darkness at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm:

30 minutes for long immunological reaction (20 hours at +4°C) and 30 to 60 minutes for short immunological reaction (3 hours at room temperature) after adding the Ellman's reagent.

Enzyme Immunoassay Protocol (Volumes are in µL)				
	Blank	**Non Specific Binding**	**Standard**	**Sample**
Buffer	**-**	**100**	**-**	**-**
Standard	**-**	**-**	**100**	**-**
Sample	**-**	**-**	**-**	**100**
Tracer	**-**	**100**	**100**	**100**
Cover plate, incubate at room temperature during 3 hours or at +4°C during 20 hours				
Wash the strips 5 times, slightly shake them 5 min, rewash them 5 times and remove the liquid from the wells				
Ellman's reagent	**200**	**200**	**200**	**200**
Incubate with an orbital shaker in the dark at room temperature				
Read the plate between 405 and 414 nm				

8.6 DATA ANALYSIS

Make sure that your plate reader has substracted the absorbance readings of the blank well (absorbance of Ellman's reagent) from the absorbance readings of the rest of the plate. If not, do it now.

_ Calculate the average absorbance for each NSB, standard and sample.

_ Plot the absorbance for each standard (Y axis) versus the concentration (X axis) using a 4-parameter logistic fitting or cubic spline fitting.

_ To determine the concentration of your samples, find the absorbance value of each sample on the Y axis. Read the corresponding value on the X axis which is the concentration of your unknown sample. Do not forget to integrate the dilution factor of your samples (due notably to the minimal dilution for the assay 1:10).

_ Samples with a concentration greater than 250 pg/mL should be re-assayed after dilution in Dilution Buffer.

_ Most plate readers are supplied with curve-fitting software capable of graphing this type of data. If you have this type of software, we recommend using it. Refer to it for further information.

9 TYPICAL DATA

9.1 EXAMPLE DATA

The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 30 minutes developing at room temperature for long immunological reaction (20h at +4°C) and 60 minutes developing for short immunological reaction (3h at RT), reading at 414 nm. A 4-parameter logistic fitting was used to determine the concentrations.

	Absorbances (mAU)	
Ghrelin standard (pg/mL)	Short immunological reaction (3h RT)	Long immunological reaction (20h +4°C)
250	2083	1880
125	1242	1020
62.5	679	539
31.3	343	277
15.6	180	135
7.81	93	63
3.91	47	34
1.95	21	18

9.2 ACCEPTABLE RANGE

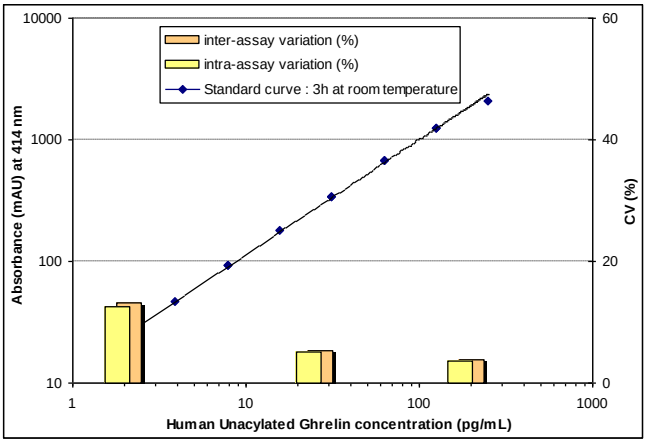
+ Non Specific Binding < 50 mAU.
+ Limit of detection in the sample before dilution: 2 pg/mL for long immunological reaction and 6 pg/mL for short immunological reaction.
+ Limit of quantification in the sample before dilution: 20 pg/mL.

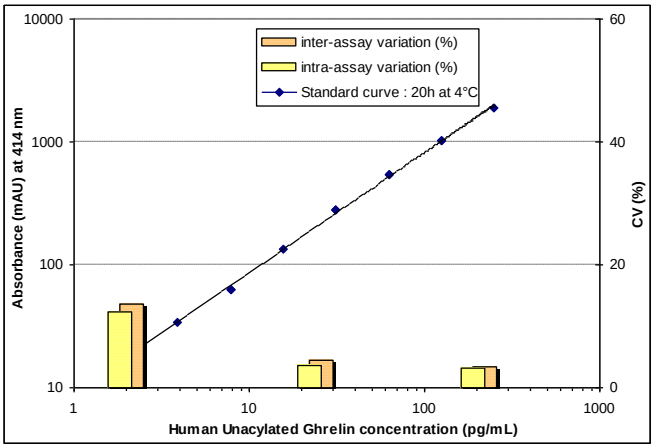
9.3 PLEASE NOTE

With Acetylcholine Esterase (AChE) tracers, enzymatic activity is batch and run dependent. This activity does not affect the sensitivity or the quality of the assay. The only restriction we put is a reading above 200 mAbsorption Units (mAU) for the highest point in order to get a significant signal to noise ratio.

If the signal looks too low for one's application, you just have to increase the revelation time until you reach the expected intensity. The plate can be read at 30 minutes, 60 minutes and 90 minutes, and so on without affecting in any way the answer. The only restriction is given by the plate reader which might not be able to read linearly after 2000 mAU.

10 HUMAN UNACYLATED GHRELIN STANDARD CURVE





11 ASSAY VALIDATION AND CHARACTERISTICS

- The Enzyme Immunometric Assay of human unacylated ghrelin has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:5). A sigmoidal 4-parameter logistic fitting was used to determine the concentrations.
- The limit of detection, calculated as the concentration of unacylated ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 0.2 pg/mL and 0.6 pg/mL respectively for long and short immunological reaction. Due to the minimal plasma dilution (1:10), the limit of detection in the samples are 2 pg/mL (20h at +4°C) and 6 pg/mL (3h at RT), respectively.

- Intra-assay & inter-assay variations and recovery:

QC levels	Theoretical concentrations in diluted QC (pg/mL)	Mean of observed concentrations (pg/mL)	Intra-assay (CV%)	Inter-assay (CV%)	Recovery (%)	Confidence intervalle (α = 0.05)
Incubation 20 hours at +4°C						
QC1	2	1.74	11.8	13.2	86.8	86.8 ± 5.8
QC2	25	21.6	3.20	3.80	86.5	86.5 ± 1.8
QC3	200	186	3.90	4.00	89.7	89.7 ± 1.4
Incubation 3 hours at room temperature						
QC1	2	1.78	12.10	12.60	88.9	88.9 ± 4.9
QC2	25	20.6	4.40	4.50	82.2	82.2 ± 1.5
QC3	200	176	4.30	4.60	87.8	87.8 ± 1.8

- The intra-assay and inter-assay variations were studied on 30 human plasma (free of ghrelin) spiked samples for each level of QC. QC were prepared 10 times concentrated from a pool of human plasma and then diluted to 1:10 in Dilution buffer before assay. Replicate samples (n=6) at each of the three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

- Matrix variability:

Matrix	Theoretical concentration (pg/mL)	Unacylated ghrelin measured (pg/mL)	Recovery (%)	Mean of recovery (%)
1	25	25.3	101	99.8
2		25.7	103	
3		25.5	102	
4		23.9	95.6	
5		24.3	97.2	

Five individual lots of human plasma samples were tested. Validation samples (n=3) were prepared five times, concentrated in each matrix (free of ghrelin) and then diluted to 1:10 in order to obtain a final concentration of 25 pg/mL. QC were analysed against a calibration curve derived from a pool of human plasmas.

- Dilution test:

Samples	Dilution factor	Unacylated ghrelin measured (pg/mL)	Corrected concentrations (pg/mL)	Recovery (%)	Mean of recovery (%)
1	1:10	66.2	662	-	98.9
	1:20	34.2	684	103	
	1:50	13.8	690	104	
	1:100	6.22	622	94.0	
	1:200	3.12	624	94.3	
2	1:10	53.2	532	-	97.5
	1:20	26.6	532	100	
	1:50	10.7	535	101	
	1:100	5.09	509	96	
	1:200	2.49	498	94	
3	1:10	64.8	648	-	96.8
	1:20	33.6	672	104	
	1:50	13.4	670	103	
	1:100	5.92	592	91.4	
	1:200	2.88	576	88.9	

Three human plasma samples were diluted to 1:10. Afterwards, four independent dilutions (n=3) were performed and analysed against a calibration curve.

- Stability test (freezing/thawing):

Samples	Reference value (pg/mL)	1 cycle (pg/mL)	2 cycles (pg/mL)	3 cycles (pg/mL)	Mean of recovery (%)
1	288	308	301	310	106
2	264	272	269	278	103
3	389	417	407	446	109
4	556	577	561	614	105
5	210	226	225	221	107

Four human plasma samples (n=3) were analysed just after collection and dilution to 1:10 before the assay (reference value) and after 1, 2 and 3 freeze/thaw cycles.

- Cross-reactivity:
- Ghrelin (Des-Octanoyl-Ser3) (Rat): 100 %
- Ghrelin (Octanoyl-Ser^{3}) (Human): <0.06 %
- Ghrelin (Octanoyl-Ser^{3}) (Rat, Mouse): <0.03 %
- Ghrelin (1-14) (Human): <0.001 %

- Ghrelin (1-11) (Rat):	<0.001 %
- Ghrelin (17-28) (Human, Rat):	<0.001 %
- GHRF (Human):	<0.001 %
- Insulin (Human):	<0.001 %
- Motiline:	<0.001 %
- Leptin (Human):	<0.001 %
- Somatostatine:	<0.001 %
- CRF (Human, Rat):	<0.001 %
- Glucagon (Human, Rat):	<0.001 %

12 ASSAY TROUBLE SHOOTING

- Absorbance values too low: incubation in wrong conditions (time or temperature) or reading time too short or Human unacylated ghrelin standard, or anti-unacylated Ghrelin tracer or Ellman's reagent have not been dispensed.
- NSB value too high: contamination of NSB wells with Human unacylated ghrelin standard, or inefficient washing.

13 REFERENCES

1. Grassi J. & Pradelles Ph. Compounds labelled by the acetylcholinesterase of Electrophorus Electricus. Its preparation process and it use as a tracer or marquer in enzymo-immunological determinations. United States patent, N° 1,047,330. September 10, 1991

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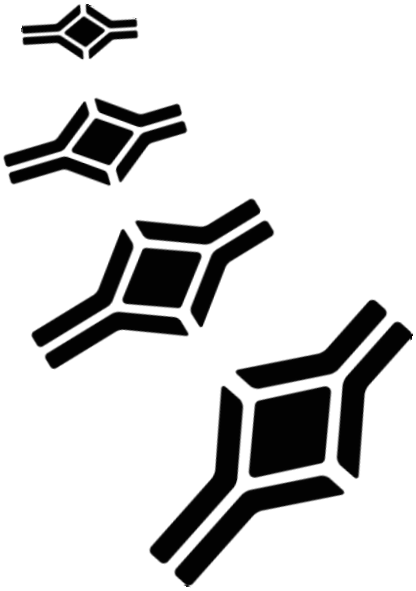

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