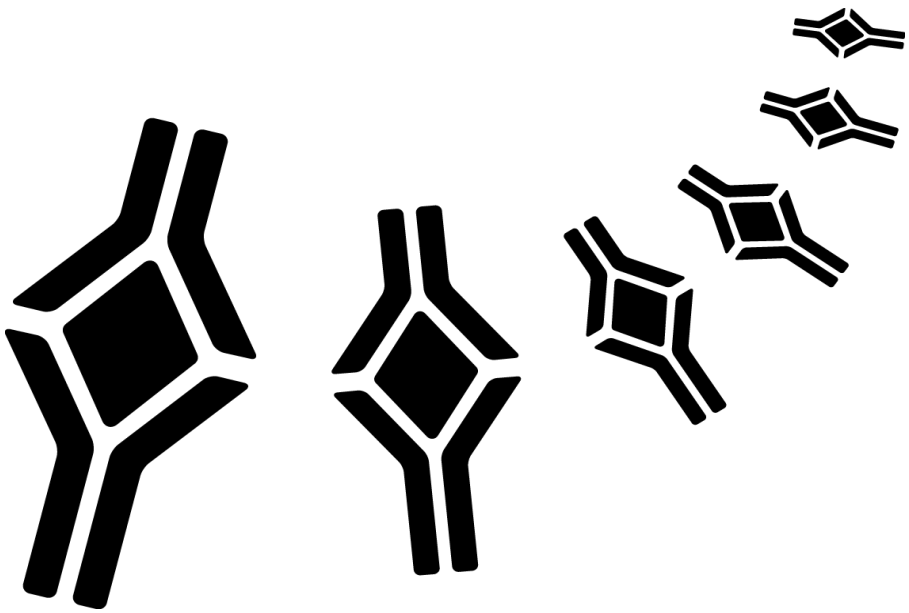


RAT ACYLATED GHRELIN ELISA

Product Data Sheet

Cat. No.: RD394062400R

For Research Use Only

CONTENTS

1	HUMAN ACYLATED GHRELIN EIA KIT	3
2	PRECAUTIONS FOR USE	3
3	PRINCIPLE OF THE ASSAY	4
4	MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED	5
5	SAMPLE PREPARATION	5
6	REAGENT PREPARATION	6
7	ASSAY PROCEDURE	7
8	TYPICAL DATA	9
9	HUMAN ACYLATED GHRELIN STANDARD CURVE	10
10	ASSAY VALIDATION AND CHARACTERISTICS	10
11	ASSAY TROUBLE SHOOTING	12
12	REFERENCES	13

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

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

1 HUMAN ACYLATED GHRELIN EIA KIT

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

This kit contains:

- A covered 96 wells plate, pre-coated with anti-Ghrelin mouse monoclonal antibody, ready to use
- One vial of Conjugate Solution (anti-acylated Ghrelin tracer), lyophilised
- Two vials of Rat acylated ghrelin standard, lyophilised
- Two vials of Quality Control, lyophilised
- One vial of Dilution Buffer, lyophilised
- One vial of concentrated 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 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.

3 PRINCIPLE OF THE ASSAY

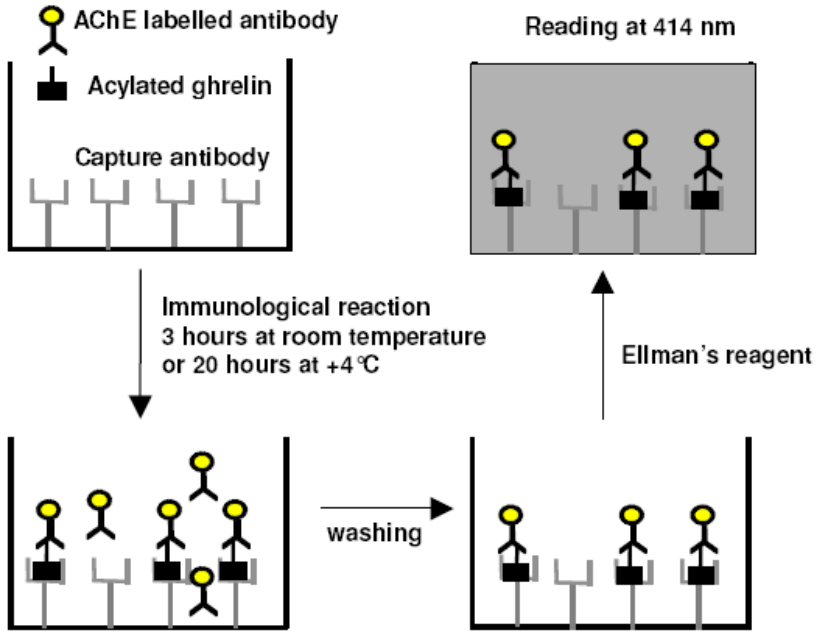
Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized principally in the stomach. It stimulates food intake and transduces signals to hypothalamic regulatory nucleid that controls energy homeostasis. The peptide consists of 28 amino acids, with a n-octanoylation of the serine-3 residue, which is necessary for the biological activity hereunder mentioned. Ghrelin is present in the peripheral circulation under two forms: acylated and non-acylated. The rat acylated ghrelin EIA kit specifically measures the acylated 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 in the wells (standard or sample). The acetylcholinesterase (AChE) - Fab' conjugate which recognises the N-terminal part of acylated ghrelin is also added to the wells. This allow the two antibodies to form a sandwich by binding on different parts of the rat acylated ghrelin.

The sandwich is immobilised on the plate so the excess reagents may be washed away. The concentration of the rat acylated 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 rat acylated ghrelin present in the well during the immunological incubation.

The principle of the assay is summarised below:



4 MATERIALS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

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

4.1 FOR SAMPLE PREPARATION

- Ethylenediaminetetra-acetic acid (EDTA)
- Potassium Phosphate buffer 0.1 M pH 7.4
- NaOH 10N
- p-hydroxymercuribenzoic acid (PHMB)
-

4.2 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
-

5 SAMPLE PREPARATION

5.1 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.**

5.2 BLOOD COLLECTION

Blood samples are collected in tubes containing EDTA and p-hydroxymercuribenzoic acid (1 mM in the final sample volume) to prevent the degradation of acylated ghrelin by protease. Samples are centrifuged at 3,500 rpm for 10 min at +4°C and then, supernatants are transferred in separate tubes. Add immediately 100 µL of 1N HCl per mL of collected plasma and centrifuge them at 3,500 rpm for 5 min at +4°C. Then, supernatants are transferred in separate tubes. Samples should be quickly assayed or stored at -20°C for later use.

For the preparation of PHMB, we suggest preparing a 100 times concentrate solution (100 mM) in potassium phosphate buffer 0.1 M pH 7.4 in which you add 1.2% NaOH 10N volume/volume. Then dissolve PHMB to get a 100 times concentrated solution (100 mM) in this buffer.

You can prepare your 100x solution several weeks before and store it in a cool place.

For assaying the unacylated ghrelin, please refer to the section “Blood collection” of the protocol of the Rat Unacylated Ghrelin EIA Kit.

5.3 SAMPLE PREPARATION

Plasma samples may be assayed directly (without any extraction procedure) after being diluted at least to 1:5 in EIA buffer in order to avoid matrix effect.

6 REAGENT PREPARATION

The coated plates and reagents are provided ready to use.

6.1 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.

6.2 Rat acylated 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 EIA 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.

6.3 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.

6.4 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.

6.5 Wash Solution Concentrate

Dilute 1 mL of concentrated Wash Solution 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.

6.6 Substrate Solution

Five minutes before use, reconstitute with 49 mL of distilled or deionized water and 1 mL of concentrated Wash Solution. The tube contents should be thoroughly mixed.
Stability at 4°C and in the dark: 1 day.

7 ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

7.1 PLATE PREPARATION

Prepare the wash buffer 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 buffer (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.

7.2 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.

7.3 PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Substrate Solution. 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 reagents.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	BI	S1	S1	QC	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
B	BI	S2	S2	QC	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
C	BI	S3	S3	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
D	BI	S4	S4	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
E	NSB	S5	S5	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
F	NSB	S6	S6	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
G	NSB	S7	S7	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
H	NSB	S8	S8	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa

B : Blank
NSB : Non Specific Binding
S1-S8: Standards 1-8
* : Samples or Quality Controls

- Dilution Buffer:

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

- Rat acylated 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 and samples:

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.

7.4 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 allow to increase the sensitivity for the assay: 0.2 pg/mL versus 0.7 pg/mL for short immunological reaction.

7.5 DEVELOPING AND READING THE PLATE

Reconstitute Substrate Solution as indicated in reagent preparation section. Wash each well five times with the wash solution (300 µL/well), slightly shake the plate for 5 minutes (with the orbital shaker) and then rewash 5 times with the wash solution (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.

7.6 DATA ANALYSIS

Make sure that your plate reader has substracted the absorbance readings of the blank well (absorbance of Substrate Solution) 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. Také care to integrate the dilution factor of your samples (due notably to the minimal dilution for the assay 1:5).
- 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.

8 TYPICAL DATA

8.1 EXAMPLE DATA

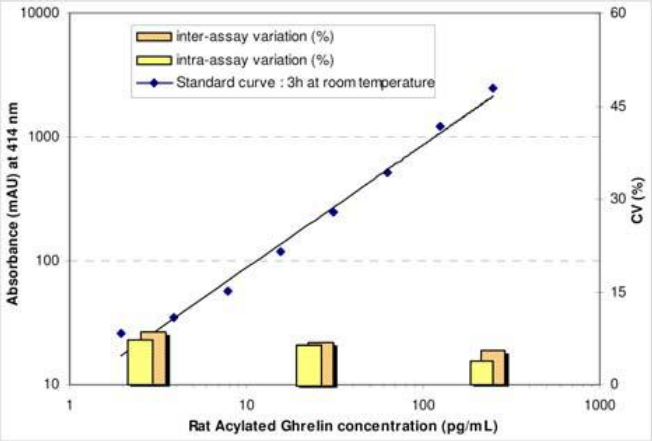
The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 30 minutes developing at room temperature for long immunological reaction (20h at +4°C) and 60 minutes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

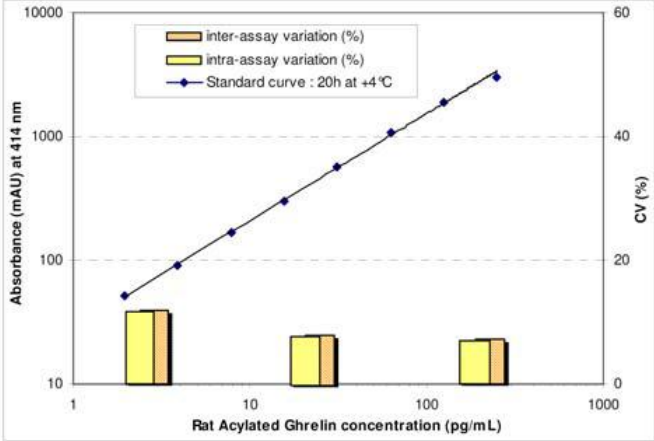
	Absorbances (mAU)	
Ghrelin standard (pg/ml)	*Short immunological reaction (3h RT)*	*Long immunological reaction (20h +4°C)*
250	2.457	3.007
125	1.214	1.897
62.5	0.514	1.084
31.3	0.247	0.571
15.6	0.118	0.304
7.81	0.057	0.166
3.91	0.035	0.090
1.95	0.026	0.051

8.2 ACCEPTABLE RANGE

- Non Specific Binding < 50 mAU
- Limit of detection in the sample before dilution: 1 pg/mL for long immunological reaction and 3.5 pg/mL for short immunological reaction.
- Limit of quantification in the sample before dilution: 10 pg/mL.

9 HUMAN ACYLATED GHRELIN STANDARD CURVE





10 ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric Assay of rat acylated 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.

10.1 The limit of detection

The limit of detection, calculated as the concentration of acylated ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 0.2 pg/mL and 0.7 pg/mL respectively for long and short immunological reaction. Due to the minimal plasma dilution (1:5), the limit of detection in the samples are 1 pg/mL (20h at +4°C) and 3.5 pg/mL (3h at RT), respectively.

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

Samples	*Theoretical concentrations in diluted QC (pg/ml)*	*Observed concentrations (pg/ml)*	*Intra-assay CV (%)*	*Inter-assay CV (%)*	*Recovery O/E (%)*	*Confidence Interval (α = 0.05)*
Incubation 20 hours at +4°C						
QC1	2	1.91	11.2	11.4	95.4	95.4 ± 4.4
QC2	25	24.9	6.8	7.0	99.5	99.5 ± 2.9
QC3	200	203	6.7	7.0	102	102 ± 3.1
Incubation 3 hours at room temperature						
QC1	2	2.12	7.0	8.1	106	106 ± 4.5
QC2	25	24.7	5.7	6.1	98.6	98.6 ± 2.8
QC3	200	190	3.7	5.4	94.8	94.8 ± 3.4

The intra-assay and inter-assay variations were studied on 30 rat plasma (free of ghrelin) spiked samples for each level of QC. QC were prepared five times concentrated from a pool of rat plasma and then diluted to 1:5 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.

10.3 Matrix variability:

Matrix	Expected *(pg/ml)*	Observed *(pg/ml)*	Recovery *(%)*	Mean of recovery *(%)*
1	25	24.6	98.4	97.7
2		23.2	92.8	
3		24.0	96.0	
4		24.8	99.2	
5		25.4	102	

Five individual lots of rat plasma samples were tested. Validation samples (n=3) were prepared five times concentrated in each matrix and then diluted to 1:5 in order to obtain a final concentration of 25 pg/mL. QC were analysed against a calibration curve derived from a pool of rat plasmas.

10.4 Dilution test

Samples	*Dilution factor*	*Acylated Ghrelin measured (pg/ml)*	*Corrected concentrations (pg/ml)*	*Recovery (%)*	*Mean of recovery (%)*
1	1:5	26.3	132	-	109
	1:10	13.9	139	105	
	1:20	7.20	144	109	
	1:25	5.68	142	108	
	1:50	3.03	152	115	
2	1:5	48.2	241	-	105
	1:10	24.4	244	101	
	1:20	12.6	252	105	
	1:25	10.2	255	106	
	1:50	5.18	259	107	
3	1:5	48.7	244	-	99.8
	1:10	25.8	258	106	
	1:20	12.9	258	106	
	1:25	9.54	239	98.0	
	1:50	4.36	218	89.3	

Three rat plasma samples were diluted to 1:5. Afterwards, four independent dilutions (n=3) were performed and analysed against a calibration curve.

10.5 Stability test (freezing/thawing):

Samples	*Expected value (pg/ml)*	*Observed 1 cycle (pg/ml)*	*Observed 2 cycles (pg/ml)*	*Observed 3 cycles (pg/ml)*	*Mean recovery O/E (%)*
1	140	129	108	102	80.7
2	268	263	225	227	88.9
3	192	183	151	152	84.4
4	221	266	222	228	108

Four rat plasma samples (n=3) were analysed just after collection and dilution to 1:5 before the assay (reference value) and after 1, 2 and 3 freeze/thaw cycles.

10.5 Cross-reactivity:

10.5 Cross-reactivity:

- Ghrelin (Human): 82 %
- Ghrelin (Des-Octanoyl-Ser^3) (Rat, Mouse): <0.001 %
- Ghrelin (Des-Octanoyl-Ser^3) (Human): <0.001 %
- Ghrelin (1-11) (Rat): <0.001 %
- Ghrelin (1-14) (Human): <0.001 %
- Ghrelin (17-28) (Human, Rat): <0.001 %
- GHRF (rat): <0.001 %
- Insulin (Rat): <0.001 %
- Motiline: <0.001 %
- Leptin (Rat): <0.001 %
- Somatostatine: <0.001 %
- CRF (Human, Rat): <0.001 %
- Glucagon (Human, Rat): < 0.001 %

11 ASSAY TROUBLE SHOOTING

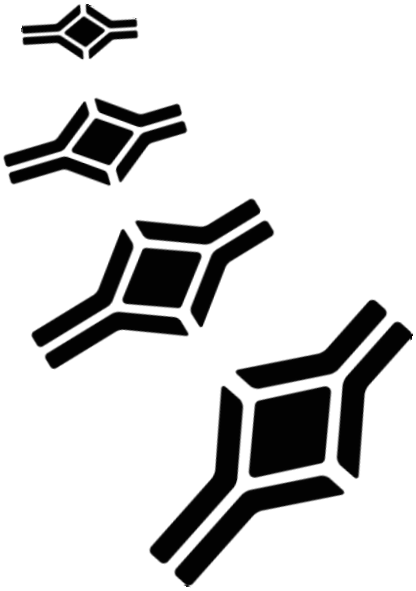
- Absorbance values too low: incubation in wrong conditions (time or temperature) or reading time too short or Rat acylated ghrelin standard, or Conjugate Solution or Substrate Solution have not been dispensed.
- NSB value too high: contamination of NSB wells with Rat acylated ghrelin standard, or inefficient washing.
- High dispersion of duplicates: poor pipetting technique or irregular plate washing.

12 REFERENCES

- 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

NOTES



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