System based on bee cells for in vitro compounds testing

BS01

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A. Kit description

Table 1. Kit contents and storage conditions:

No.	Component	Ilość	Storage conditions
1.	Bee ovary cells	3 vials (KP)	-80°C
2.	Vial Z1: medium supplemented with a compound with a defined influence on the viability of bee cells (concentration of the test compound in the medium: 0.1 mM), Vial K+ (possitive control): medium non-supplemented Vial K- (negative control): medium supplemented with 10% ethanol.	3 vials (Eppendorfs) Z1 – yellow (1 ml) K+ - green (1 ml) K- - blue (1 ml)	4°C
3.	HBSS/*Hank's Balanced Salt Solution*)	30 ml Falcon tube	4°C
4.	Trizol	5 ml (centrifuge tube – Falcon)	4°C
5.	Glycogen	5 µl (light blue tube 0.2 ml)	-20°C
6.	Chloroform	1 ml (Eppendorf)	
7.	Isopropanol	2 ml (centrifuge tube – Falcon)	4°C
8.	Ethanol 70 %	10 ml (centrifuge tube – Falcon))	4°C
9.	Kit 1	A (colorless tube 0.2 ml) B (orange tube 0.2 ml)	-20°C
10.	Kit 2	A (yellow tube 0.2 ml) B (pink tube 0.2 ml) C (green tube 0.2 ml) D (violet tube 0.2 ml) E (blue tube 0.2 ml)	-20°C
11.	Specific oligonucleotides (10 µM) mixture (sense + antisense primer)	10 µl (yellow tubes 0.5 ml)	-20°C
12.	Kit 3	1 ml (Eppendorf tube)	4°C
13.	UltraPure DEPC-Treated Water	1,5 ml (Eppendorf tube)	4°C

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B. Equipment and reagents needed for the procedure but not supplied with the kit:

- High purity water for PCR
- Eppendorf tubes 1.5 ml
- PCR tubes (single / in strips) / 1.5 ml PCR plates
- Tube racks
- Sterile pipet tips
- Adjustable automatic pipettes
- Microcentrifuge
- Vortex
- Heating block / water bath
- CO_2 Incubator (32°C / 5% CO_2)
- Real time thermal Cycler

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C. Procedure for testing bioactive compounds using bee cells

C1. Tests of bioactive compounds using bee cells

1. Centrifuge vials containing bee cells - 3 minutes / 300 × g.

2. Gently remove a medium, watch to do not move the cells in the bottom of the vials.

3. Add 500 µl of medium supplemented with test compound (Z1), non-supplemented medium (K +) and medium supplemented with 10% ethanol (K-) to each of the vials with cells.

4. Suspend the cells in the culture medium (by gentle pipetting).

5. Incubate bee cells at 32°C, 90% humidity and 5% CO_2 for 2 hours.

6. After incubation, centrifuge cells (3 minutes / 300 × g). Remove the supernatant.

7. Suspend the cells in 200 µl of Hanks salt solution containing no magnesium and calcium ions. Spin the cells again (3 minutes / 300 × g) and remove the supernatant after centrifugation. Isolate RNA from cells (according to the protocol in point C2.1.)

C2. Evaluation of the biological activity of compounds using RT-qPCR (*Reverse Transcriptase quantitative Polymerase Chain Reaction*).

The biological activity of compounds is evaluated on the basis of changes in transcript levels of five pluripotent markers, vitellogenin (VTG) and its receptor (VTGR) (7 markers).

C2.1. Izolacja całkowitego RNA metodą chloroformowo-fenolową

1. Lyse bee cells obtained in point C1.7. Add 1 ml of Trizol (Tri® Reagent) to each tube, mix thoroughly, incubate 7 minutes at RT (room temperature).

2. Add 200 µl of chloroform to the samples and shake them thoroughly for 10 seconds. Leave the samples for 7 minutes at RT.

3. Centrifuge the samples for 15 minutes (12 000 × g / 4ºC).

4. Transfer the aqueous phase to the new Eppendorfs.

5. Add 1 µl glycogen (UltraPure ™ Glycogen) and 600 µl isopropanol to the aqueous phase. Incubate the samples for 7 minutes at RT.

6. Centrifuge the sample for 1 hour (12 000 x g / 4ºC).

7. Remove the supernatant. Rinse the precipitate twice using 1 ml 70% ethanol in each case. After each rinse, centrifuge the samples for 5 min (7 500 × g / 4°C).

8. Leave the precipitate to dry at RT (about 5 minutes).

9. After drying, dissolve the precipitate in 30 µl of UltraPure DEPC-Treated Water.

10. Perform a quantitative and qualitative analysis of the RNA (spectophotometric measurement, electrophoretic separation).

C2.2. Synthesis of complementary DNA (cDNA)

1. Before proceeding with the synthesis of cDNA, digest the remaining genomic DNA using Kit 1: incubate 1 µg of total RNA in 24 µl of the reaction mixture containing reagent A (colorless tube) - 1x concentrated buffer for DNase (2.4 µl) and reagent B (orange tube) - 100x concentrated DNase (0.24 µl) for 10 minutes at 30°C. Then deactivate the enzyme at 55°C for 5 minutes.

2. Use a gDNA-free RNA assay for cDNA synthesis by reverse transcription using Reaction Kit 2. Perform the reaction in a total volume of 40 µl using oligo(dT)probes. The composition of the reaction mixture is shown in Table 2.

Table 2. The composition of the reaction mixture of the complementary DNA synthesis reaction.

	Component of the reaction mixture	
1.	Total RNA (DNA free)	1 µg in 24 µl of reaction mixture (obtained after digestion with DNase)
2.	A (yellow vial 0.2 ml) - oligo(dT)probes	2 µl
3.	B (pink vial 0.2 ml) - 10 mM dNTP mixture	2 µl
4.	C (green vial 0.2 ml) 5x buffer for RT	8 µl
5.	D (violet vial 0.2 ml) RNase inhibitor	2 µl
6.	E (blue vial 0.2 ml) reverse transcriptase	2 µl

Incubate the samples at 45°C for 30 minutes. Then deactivate the enzyme by incubating the samples at 85°C for 5 minutes.

C2.3. Preamplification of matrices

1. Perform the reactions in a total volume of 40 µl using Kit 3. The composition of the reaction mixture is shown in Table 3.

Table 3. The reaction mixture for preamplification of the matrices.

	Component of the reaction mixture	
1.	cDNA (obtained in step C.2.)	4 µl
2.	7 sets of primers (10 µM) (F+R)	0,1 µl each
3.	Kit 3 (2x)	20 µl
4.	UltraPure DEPC-Treated Water	15,3 µl

** The specificity of PCR products was confirmed by analyzing the dissociation curve of amplicons. The melting curves were generated by raising the reaction temperature from 65°C to 95°C at a heating rate of 0.2°C/s and continuing the fluorescence measurement. The specificity of the products was also verified against NTC (no template control) and NRT (no reverse transcriptase control). The relative amount of transcripts was calculated using the 2- ΔΔCT method. The calculations were made according to the formula (Livak and Schmittgen, 2001):

ΔΔCT = ΔCT (sample tested) - ΔCT (reference sample);

CT (sample tested) = CT of the tested gene - CT of the reference gene;

ΔCT (reference sample) = CT of the tested gene - CT of the reference gene.

The reference sample consisted of cultures not treated with tested compunds. β-actin was selected as the reference gene.

The protocol includes:

a) initial denaturation at 95°C for 2 minutes;
b) 18 repeating cycles, each consisting of denaturation (95°C/5 seconds), primer attachment (62.2°C/3 minutes) and extension of reaction products (72°C/5 seconds).

C2.4. Quantitative polymerase chain reaction/qPCR

1. The reaction should be performed in a final volume of 10 µl using the Kit 3. For a single qPCR reaction, use 1 µl of each matrix obtained in the preamplification step and specific 7 sets of primers (10 µM) (F+R) at a final concentration of 0.5 µM.

Tabela 4. The qPCR reaction mixture.

	Component of the reaction mixture	
1.	Reaction mixture after preamplification (product obtained in step C.3.)	1 µl
2.	7 sets of primers (10 µM) (F+R)	0,5 µl
3.	Kit 3 (2x)	5 µl
4.	Water	Do 10 µl

The protocol includes:

a) Initial denaturation at 95°C for 2 minutes;
b) 40 cycles consisting of denaturation (95°C/5 seconds), primer hybridization (62.2°C/10 seconds) and elongation (72°C/5 seconds);
c) analysis of melting curves of products - generate melting curves by raising the reaction temperature from 65 ° C to 95 ° C at a heating rate of 0.2 ° C / s and continuing the fluorescence measurement.