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Integrated Protocol for ROS/RNS, Glutathione, Enzyme Activity, qPCR, and Flow Cytometry Analyses in HCT-116 Cells

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Redox Biology and RDI



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We use this protocol and it's working

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Disclaimer

This protocol describes laboratory procedures performed under controlled research conditions. Users should adapt the steps to their local laboratory safety regulations and institutional guidelines.

Abstract

This protocol provides a unified workflow for quantitative redox profiling of HCT-116 cells using a comprehensive panel of biochemical, molecular, and cytometric assays. It integrates standardized methods for determining ROS and RNS levels (NBT, hydrogen peroxide, Griess assay), non-enzymatic antioxidant status (GSH/GSSG), antioxidant enzyme activities (SOD, GPx, GR, GST), relative gene expression (qPCR), and protein-level detection by flow cytometry. The procedures are optimized to generate reproducible, high-resolution measurements of cellular redox homeostasis under treatment with Gold(III) complexes. This integrated protocol supports reproducible laboratory workflows and provides a complete experimental foundation for downstream computational analyses, including the calculation of the Redox Disturbance Index (RDI).



Materials

DMEM (Dulbecco's Modified Eagle Medium), FBS (fetal bovine serum), PBS (phosphate-buffered saline), penicillin/streptomycin, trypsin-EDTA, DMSO (dimethyl sulfoxide), human colon carcinoma cell line (HCT-116), Gold(III) complexes AK1 and AK2, NBT, KOH solution, DMSO, 1% sulfanilamide, 1% NED solution, Phenol red, HRPO enzyme type 2, sodium hydroxide solution, 5,5'-dithiobis (2-nitrobenzoic acid) - DTNB, 0.3M Na₂HPO₄, 4-vinylpyridine, NADPH, phosphate buffer, UV-Vis spectrophotometer, TRIzol reagent, chloroform, isopropanol, ethanol, RNase/DNase-free water, NG dART RT Kit (Cat. No. E0801-02, EURx Molecular Biology Products), FastGene IC Green 2 x qPCR Universal Mix (Cat. No. LS41, NIPPON Genetics EUROPE, Düren, Germany), MIC qPCR Cycler (Biomolecular Systems, Yatala, Australia), Thermo Fisher Scientific primers, Gyrozen high-speed centrifuge, 1248R model (Daejeon, Korea), Multiskan SkyHigh UV/Vis Spectrophotometer with μ Drop Duo Plate (Thermo Fisher Scientific), CitoFix solution, PermWash solution, HIF1 alpha Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-13555, lot: EC8713, Elabscience), NFkappaB-p65 Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-32232, lot: DK7552, Elabscience), NRF2 Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-63485, lot: EC0720, Elabscience), PE Donkey anti-rabbit Polyclonal IgG (minimal x-reactivity) Antibody (Cat.No: 406421, lot: B326433, BioLegend), BD FACSVerser Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Cell preparation and culture conditions

- 1 The human colon carcinoma cell line (HCT-116) was obtained from the European Collection of Authenticated Cell Cultures (ECACC). After defreezing, the cells were grown in complete medium – DMEM, 10% fetal bovine serum, antibiotics 100 IU/mL penicillin and 100 µg/mL streptomycin, in 75 cm² culture flasks, on a controlled atmosphere of 5% CO₂ at 37 °C (Langdon, 2004). 2-3 passages after defreezing, at confluence 70-80%, cells were ready for experiments.
- 2 Gold(III) complexes AK1 and AK2 were tested in 4 doses (0.1 - 1 - 10 - 50 µM) in assays of quantifying ROS and RNS, of non-enzymatic antioxidants and antioxidant enzyme assays, while they were tested in 2 doses (1 and 10 µM) in gene and protein expression studies (AK1-1, AK1-10, AK2-1, AK2-10).

Assays of quantifying ROS and RNS

- 3 NBT assay: determination of superoxide anion radical - The spectrophotometric assay for determining the superoxide anion radical, O₂⁻, is based on measuring the absorbance of nitroblue-formazan resulting from the reduction of NBT in the presence of O₂⁻ (Petrović et al, 2021; Auclair and Voisin, 1985). In this assay, 10⁴ cells were seeded in 96-well plates and after 24 h were treated with 100 µL of the investigated substances in a final concentration range from 0.1 to 500 µM. The O₂⁻ was estimated 24 and 72 h after treatment by adding 10 µL of NBT (5 mg/mL PBS) and 100 µL of complete medium, incubated under controlled conditions for 3 h. Then, the cells were washed with PBS, while the colored biochemical reaction was determined spectrophotometrically at 630 nm after adding 100 µL of KOH solution and 100 µL of DMSO. Untreated cells served as a negative control.
- 4 Griess assay: determination of nitrites - Spectrophotometric detection of nitrites, which could be considered indicators of nitric oxide (Petrović et al, 2021; Griess, 1879), is based on the diazotization reaction of nitrites and sulfanilamide and NED. A purple diazo product was spectrophotometrically detected at 492 nm. Similarly, to NBT assay, 10⁴ cells were seeded in 96-well plates with treatment, and after 24 and 72 h nitrite measuring was performed. The procedure involves using 50 µL of supernatant from 96-well plates, adding 50 µL of freshly prepared 1% sulfanilamide (dissolved in 5% phosphoric acid) and 50 µL of freshly prepared 0.1% NED solution. Such a prepared reaction mixture is measured on an ELISA reader, and untreated cells were considered as a control.
- 5 The determination of hydrogen peroxide (H₂O₂) – Concentration was based on the oxidation of phenol red in the presence of horseradish peroxidase as a catalyst. Detached cells were counted, lysed, and the supernatant was separated. 100 µL of reaction mixture containing Phenol red and HRPO enzyme type 2 was added, and

allowed to incubate for 1 hour at 37°C. The reaction was stopped with 10 µL of sodium hydroxide solution, and the absorbance was measured at 600 nm (Petrović et al, 2021; Pick and Keisari, 1980).

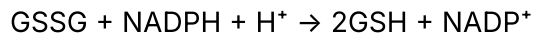
Assays of non-enzymatic antioxidants

- 6 Reduced glutathione (GSH) was determined using the method of Rahman et al, based on GSH oxidation with 5,5'-dithiobis (2-nitrobenzoic acid) - DTNB. Detached cells were counted, lysed, and the supernatant was separated. 160 µL of 0.3M Na₂HPO₄ and 20 µL of 0.04% DTNB were added to 20 µL of supernatant. Yellow product, 5'-thio-2-nitrobenzoic acid, is measured at 405 nm after 10 min of incubation period an ELISA reader. Untreated cells were considered as a control.
- 7 The concentrations of oxidized glutathione (GSSG) were determined after enzymatic reaction with glutathione reductase (Rahman et al, 2007). Detached cells were counted, lysed, and the supernatant was separated. In 50 µL of supernatant, 1 µL of 4-vinylpyridine was added first, and then 100 µL of master mix containing the enzyme glutathione reductase, NADPH, DTNB, and phosphate buffer, pH 7.4, was added. After 10 min of incubation, the absorbance was measured at 405 nm on ELISA reader. Untreated cells were considered as a control.

Antioxidant enzymes assays

- 8 These tests were preceded by cell preparation. After treatment in T25 flasks, cells were transferred to suspension and counted. Cells were then lysed for 30 minutes in the freezer, and the lysate was used for further work.
- 9 Enzyme activity was expressed as the change in absorbance per unit time per milliliter of cell suspension. Absorbances were measured with a UV-Vis spectrophotometer at certain wavelengths for each enzyme separately. The absorbance of the sample compared to the absorbance of the blank sample was used to calculate enzyme activity.
- 10 Superoxide dismutase (SOD) activity was determined using the method of Marklund and Marklund (1974), based on ability of SOD to inhibit spontaneous autoxidation of pyrogallol by superoxide anion radical (O₂⁻) in alkaline environment. Activity was measured with UV-Vis spectrophotometer at 420 nm. To calculate SOD activities, sample absorbance compared to the sample absorbance of blind probe is used.
- 11 Glutathione peroxidase (GPx) activity was determined by method of Maral et al (1977). The principle of the method is based on ability of GSH-Px to catalyze the oxidation of GSH to GSSG and reduction of the large amounts of hydroperoxide. Absorbance was measured with UV-Vis spectrophotometer at 340 nm, compared to a blind probe.
$$2\text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}$$
- 12 The activity of glutathione reductase (GR) was determined using the method of Glatzle et al (1974). The method is based on the capacity of GR to catalyze the reduction of GSSG

to GSH using nicotinamide adenine dinucleotide phosphate (NADPH) as substrate for simultaneous oxidation to NADP⁺. Absorbance was measured with UV-Vis spectrophotometer at 340 nm, compared to a blind probe.



- 13 Glutathione-S-transferase (GST) activity to catalyze the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and sulfhydryl group (-SH) of reduced glutathione was measured according to the method of Habig et al (1974). Change of absorbance was measured with UV-Vis spectrophotometer at 340 nm, compared to a blind probe.
- $$\text{CDNB} + \text{GSH} \rightarrow \text{CDNB-S-glutathione}$$

Relative gene expression (qPCR)

- 14 qPCR assays of relative gene expression were performed according to laboratory protocols mainly based on MIQE Guidelines (Bustin et al, 2009) and in accordance with GLP propositions for PCR detection and sample-centric laboratory manipulation propositions.
- 15 Sample manipulation - All RNA extractions and concentration estimation were performed immediately after sampling. For each group of isolated RNA, reverse transcription was performed straightaway. cDNA was stored at -20°C until all samples were prepared and ready for qPCR relative gene expression detection.
- 16 RNA extraction – Total RNA was extracted in 1 ml of TRIzol reagent (Thermo Fisher Scientific) and 0.2 ml chloroform according to the protocol. After separating the three phases, we take the upper phase containing the total RNA for further work. RNA is precipitated using 0.5 ml of isopropanol and then washed twice in 1 ml of 70% ethanol. Then, the obtained RNA is dried on the Thermoblock (HB120-S, DLAB Scientific), and finally eluted and maintained in RNase/DNase-free water. To RNA extraction, the Gyrozen high-speed centrifuge, 1248R model (Daejeon, Korea) was used.
- 17 RNA quantification - The RNA concentration was estimated on Multiskan SkyHigh UV/Vis Spectrophotometer with $\mu\text{Drop}^{\text{TM}}$ Duo Plate (Thermo Fisher Scientific) on 260 nm. The yield was optimal with A_{260}/A_{280} ratio between 1.75 to 2.0.
- 18 Reverse transcription was performed by using of NG dART RT Kit (Cat. No. E0801-02, EURx Molecular Biology Products). According to manufacturer proposition, the isolated and quantified RNA was immediately reversal transcribed. The kit contains modified reverse transcriptase with improved thermostability (up to 65°C) and processivity for synthesizing the cDNA strands. The including reagents prove the minimization of RNase H activity and processivity. Total RNA template was added in concentration of 1.5 μg in 1 μL oligo dT primer, 1 μL NG dART RT Mix, 4 μL 5 x NG cDNA Buffer and RNase-free water up to 20 μL . The reaction mixture was incubated at 50°C for 60 minutes at Mic qPCR Cycler (Biomolecular Systems, Yatala, Australia), followed by final incubation at 85°C for 5 min for enzyme deactivation.

- 19 qPCR primers - Primers for qPCR reaction were designed and purchased from Thermo Fisher Scientific. Primers were DSL purified and C18 desalinated, no-end modified.
- 20 qPCR protocol - For the purpose of qPCR reaction for determination of relative gene expression, the FastGene IC Green 2 x qPCR Universal Mix (Cat. No. LS41, NIPPON Genetics EUROPE, Düren, Germany) was used. This kit is based on using of FastGene IC Green as an intercalating dye, only detecting double stranded DNA. According to manufacturer supporting information, 100 ng of cDNA was mixed with 10 μ l 2X FastGene IC Green, 0.8 μ l of forward and 0.8 μ l of reverse primer (10 μ M) with addition of PCR-grade water up to 20 μ l qPCR reaction. Mic qPCR Cyclers (Biomolecular Systems, Yatala, Australia) were used for cycling reaction with additional high resolution melting analyses. Initial denaturation was performed at 95°C for 2 minutes. Additional 40 cycles included two steps: Denaturation at 95°C for 5 seconds and annealing and elongation at 62°C for 30 seconds. Melting analyses were performed at the end of 40 cycles as the proof of reaction product procedure, and it was shown that there were no impurities, and neither were the primer dimer peaks. Every qPCR run also analyzed NTC control reaction. MIC qPCR Cyclers use specially designed ultra clean tubes preloaded with a high viscosity silicon oil for evaporation exclusion. Dual filter pipette tips were used for all work in the laboratory (Ptasinska et al, 2021; Matic et al, 2023).
- 21 Data analyses - MIC qPCR Cyclers are controlled by intuitive software package micPCR v2.10.3 for obtaining Ct values and subsequent melting analyses. Because it has proved that there is no single gene that is constitutively expressed in all cell types (Andersen et al, 2004), for research on cancer cells we choose β -actin as reference gene (Yang et al, 2020). After data analysis, the relative gene expression was calculated by using delta-delta Ct method formula according to Livak and Schmittgen (Livak and Schmittgen, 2001). The results were calculated as the mean value of triplicate for each sample and were presented as mean fold change $\pm$ standard deviation (SD) for each gene for treated cells compared to healthy control (value set to 1).

Flow cytometry - Protein detection

- 22 For quantitative determination of proteins related to redox balance, flow cytometry analysis was performed. (Baskić et al, 2006; Živanović et al, 2023) The cells were cultured in complete medium treated with Au-complexes as previously described. After 24 and 72 h, the cells were detached with trypsin-EDTA, and the samples were transferred to flow cytometry tubes.
- 23 After washing with PBS, the tubes were centrifuged at 400 g for 5 min, and the cells were fixed with CitoFix solution for 20 min. Then, the cells were washed with PermWash solution, incubated with specific primary monoclonal antibodies for 30 min. The monoclonal antibodies used are:



HIF1 alpha Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-13555, lot: EC8713, Elabscience),
NFkappaB-p65 Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-32232, lot: DK7552, Elabscience),
NRF2 Rabbit Polyclonal IgG Antibody (Cat.No: E-AB-63485, lot: EC0720, Elabscience),
The cells were washed again, incubated with fluorochrome-conjugated secondary monoclonal antibodies for 30 min, and re-suspended in PBS. Proteins of interest were labeled with PE Donkey anti-rabbit Polyclonal IgG (minimal x-reactivity) Antibody (Cat.No: 406421, lot: B326433, BioLegend). The cell suspensions were analyzed on BD FACSVerse Flow Cytometer (BD Biosciences, San Jose, CA, USA). (Živanović et al, 2023)

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