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Standard Operating Procedure

Title	***Standard Operating Procedure (SOP) for TaqMan real-time Reverse Transcription PCR***
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Standard Operating Procedure (SOP) for TaqMan real-time Reverse Transcription PCR

1. Background

Reverse transcription-polymerase chain reaction (RT-PCR) is a relatively rapid, sensitive and simple technique to determine the mRNA expression level of different target genes and is widely used in biomedical research including toxicity studies. Real-time PCR allows for the detection of PCR amplification in the exponential growth phase of the reaction and is much more quantitative than traditional RT-PCR. Reverse-transcribed RNA (cDNA) is used as the template for amplification and combined with TaqMan probe for detection. TaqMan PCR which is Probe-based real time PCR requires a pair of PCR primers and an additional fluorogenic oligonucleotide probe with both a reporter fluorescent dye and a quencher dye attached. Here, we describe the procedures for total RNA isolation by using Maxwell® total RNA isolation kit, for reverse transcription (RT) by High capacity cDNA Reverse Transcription Kit. And real-time PCR will be performed on a StepOnePlus real-time PCR detection system (Applied Biosystems) by using TaqMan probe-based method (Figure 1).

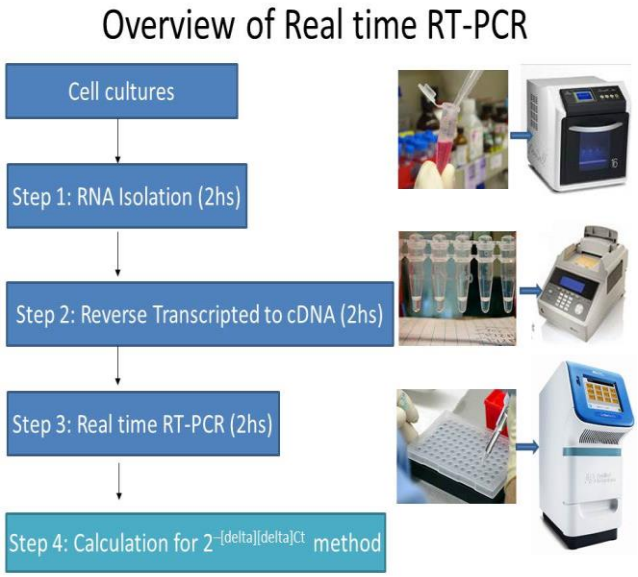

Figure 1: Four steps flow chart for real time RT-PCR: First, collect the cell cultures after exposure; then total RNA isolation by Maxwell LEV RNA kit and reverse transcription reaction to cDNA; next run the amplification in StepOne Plus system; finally calculate the relative mRNA expression.

2. Protocol

2.1. Cell culture, nanomaterial exposure, and sample preparation

- Lung epithelial cancer (A549) cells were generously provided by the project partner NILU, which were shared by all the partners in task 5.6 (cell line originally from ATCC)
- Human gingival fibroblasts (GF) were collected after informed consent from healthy patients who underwent extraction of wisdom teeth at the Department of

Clinical Dentistry in Bergen (IKO, UiB) (ethical approval was obtained before the project start)
- Nanomaterial batch dispersion (2.54 mg/mL)
- Pipette and pipette tips (sterile)
- Glass Pasteur pipettes (sterile)
- Trypan blue solution, 0.4% (InvitrogenTM, Molecular ProbesTM)
- CountessTM cell counter and cell counting chamber slides (InvitrogenTM, Molecular ProbesTM)
- Phosphate-buffered saline (1x PBS, Life Technologies/Gibco)
- Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ Supplement (Life technologies/Gibco) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) (GE Healthcare - HyClone™) and 1 % (v/v) Penicillin-Streptomycin (P/S) (10.000 UI/mL penicillin and 10.000 µg/mL streptomycin) (GE Healthcare - HyClone™)
- NuncTM cell culture treated EasYFlasksTM: 175 cm2 (Thermo Scientific)
- NuncTM cell culture treated Multidishes: 6-well dish (Thermo Scientific)
- Falcon tubes sterile, 15 and 50 mL (Sarstedt AG & Co)
- Nikon Eclipse TS100 inverted microscope (10x NA 0.25 and 40x NA 0.55)
- Laboratory liquid suction system with vacuum pump
- Tube rotator SB3 (Bibby Scientific Limited)
- Sterile hood

Cell culture

A549 and GF cells cultured in DMEM (+additives) were grown in cell culture flasks: 75 cm^2 in 15 mL cell culture medium or 175 cm^2 in 25 mL cell culture medium. The medium was changed every 2-3 days and cells were trypsinised when a confluency of 80% was reached. All cells were kept at 37°C in a humidified, 5% CO2 atmosphere.

For the experiments, cells were used if their viability was above 90% (measured by Trypan blue exclusion, see below) and underwent no more than 15 passages. After reaching 80% confluency, cells were washed two times with PBS and detached from the flasks using trypsin (2.5 mL for 5-10 min). Next, 5 mL of cell culture medium were added and the cell suspensions were transferred into 15 mL Falcon tubes. Cells were collected by centrifugation at 250 g for 5 min. The supernatant was carefully removed and the cell pellet was re-suspended in cell culture medium. Subsequently, cells were counted using trypan blue dye exclusion and the Countess automatic cell counter, then cell culture medium was added to obtain a stock suspension at a density of 10.000 cells /well for GF (corresponds to 50.000 cells/cm^2) and 5.000/well for A549 cells (corresponds to 25.000 cells/cm^2) so that at the time of exposure to NMs (24 hrs after seeding) both cell types had similar cell densities.

Nanomaterial dilution in medium and cell exposure

The stock dispersion of the NMs was prepared as specified in the generic NANOGENOTOX protocol resulting in a 2.56 mg/mL stock dispersion. A 130 watt probe sonicator (VCX130, Vibra-Cell, 130W, Sonics & Materials) with a 12.8 mm probe with a replaceable tip was used for 16 min with 22% of the maximal amplitude. For a more detailed description see the NANOGENOTOX dispersion protocol.

After sonication, a 100-µg/mL-dispersion in media was prepared by adding the calculated amount of stock dispersion to the medium (e.g., 1.76 mL batch dispersion in 43.24 mL media) and mixed using a tube rotator at 40 rpm for 2 min (outside the sterile bench).

Subsequently, a dilution row was prepared resulting in the following NM-concentrations: 100, 50, 20, 10, and 2 µg/mL (Figure 2).

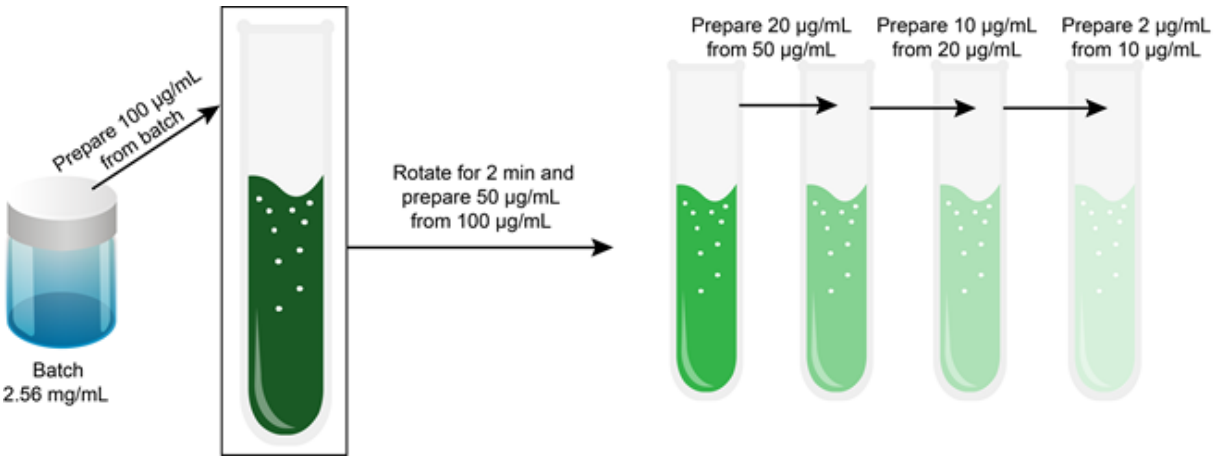

Figure 2: Preparation of NMs in medium for cell exposure using five different concentrations.

After 24 hrs, the supernatant was removed and NMs suspended in cell culture medium were added at concentrations of 2, 10, 20, 50, and 100 µg/mL corresponding to 1, 5, 10, 25, and 50 µg/cm^2 in a total volume of 4.75 ml /well.

Sample preparation

1. After 24 hrs of exposure to 4.75 mL NM-containing media, cells seeded in 6-well plates were centrifuged at 1000 g for 5 min at room temperature. In order to avoid RNA degradation, the 6 well-plates were kept on ice and the medium carefully removed. Carefully, 200 µl 1-Thioglycerol/Homogenization Solution from Maxwell 16 LEV RNA kit were loaded to cells and then incubated at room temperature for 5 min. Using a 1 ml pipet tip the whole surface of 6-well-plates was scraped and then all the solution collected in a 1.5 ml nucleanase free-tube.

The whole 6 well-plate was stored at -80 before RNA isolation.

2.2. Total RNA isolation, cDNA synthesis and real time PCR

- Reagents:

  Maxwell® 16 LEV simplyRNA Cells Kit (AS1270)

  High capacity cDNA Reverse Transcription Kit from ABI (4368814)

  TaqMan® Fast Universal PCR Master Mix from ABI (4352042)

  TaqMan® Gene Expression Assays from ABI

- Instruments:

  StepOnePlus® PCR machine from ABI

  GeneAmp PCR 9700

  Maxwell® 16 Instrument from Promega

  Nanodrop 1000 from Thermo Scientific

  Micropipettors and tips, 10 ul, 100 ul and 1 ml

1.5 ml DNase/Rnase-Free tubes

Vortex

Fast 96-well PCR reaction plates

Plate Centrifuge

Step 1: RNA isolation

Note: prepare an ice box before Isolation

1. Thaw homogenates in 1.5 ml tube on ice to avoid RNA degradation. Shortly before processing samples on the Maxwell® 16 Instrument, add 200µl of Lysis Buffer.
2. Vortex for 15 sec to mix and then keep it on ice.
3. Setup for Maxwell 16 instrument:
    - Turn on the instrument, verify "LEV" mode
    - Select "Run" on the Menu and "SimplyRNA", then after "OK" open the door
    - Transfer the rack to load all the cartridges, plungers and elution tubes
    - Add 5µl of DNase to well 4 (color will be green).
    - Transfer 400 µl of lysate to well 1 of the cartridge
    - Add 40 µl nuclease-free water to elution tubes
    - Press "Run" to start
4. End of Run: remove rack from instrument and measure RNA quantity by Nanodrop 1000. Then store RNA samples at -80°C.

Step 2: Reverse transcription (RT) reaction for cDNA synthesis:

1. Allow the kit and RNA to thaw on ice.
2. According to the concentration measured by Nanodrop, prepare 10 µl RNA solutions which contain 300 ng total RNA in Nuclease free water (use 8 strip tubes).
3. Note: prepare the RT mix on ice as the table below:

Volume (ul)	Component
10	Total RNA solution
2	10x RT buffer
0.8	25x dNTPs Mix
2	10x RT Random primers
1	MultiScribe reverse transcriptase
4.2	Nuclease-free water
20	Total

4. Pipette up-down to mix samples and briefly centrifuge the tubes to spin down the contents and to eliminate any air bubbles.
5. Load the reaction tubes to GeneAmp PCR 9700 and run the program as follows:

	Stage 1	Stage 2	Stage 3	Stage 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

6. End of Run: take out the cDNA and add 90 µl of Nuclease-free water. Keep the samples on ice for the next process.

Step 3: PCR reaction

1. Prepare the PCR mix: It is advisable to run one plate per pair of endogenous/target assays to simplify set-up. Determine the number of samples (plus controls) that will be run simultaneously on one plate. Carefully design the layout for 96-well-plate and prepare the reaction mix as follows in a Fast 96 well PCR reaction plate:

Volume	Components
1.0 µl	cDNA template
5.0 µl	Fast Master Mix
0.5 µl	Gene assay
3.5 µl	H_2O
10 µl	Total

2. Gently mix the PCR mix by inversion and transfer 9 µl mix to each well and load 1 µl l cDNA afterwards.
3. Seal the plate tightly with film and centrifuge the plate at 1000g for 2 min.
4. Place the reaction plate in the instrument when instructed by software. Specifically, the operating conditions with "Fast Taqman" are as follows:

Cycles	Duration of Cycle	Temperature
1	20 seconds	95°C
40	3 seconds	95°C
	30 seconds	60°C

5. End of Run: insert an USB disc to collect the results.
6. Use StepOne software for calculations. Following amplification, compare CT values of samples (normalized to internal standard) in order to assess fold differences in mRNA levels of the target genes.