Tox-Omics

qPCR Prediction Protocol for Simple Detection of Liver Carcinogenicity in rat

Ver. 17.02

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1. ANIMAL TEST

1.1. General

Normally, generally conducted animal tests are designed according to the test purpose, and various examination measures (e.g., hematology, clinical biochemistry, and histopathology) are included. By introducing gene expression analysis into conventional animal tests, it is possible to obtain extremely valuable information (i.e., exhaustive biological responses) that has long been unavailable using traditional examination measures.

In this chapter, we describe basic remarks to be noted in animal experiments aimed at implementing the qPCR prediction method.

By considering the following basic remarks, this qPCR prediction method may also be applied to liver samples obtained from animal tests according to OECD Test Guideline 407[1] or the 28-day repeated-dose toxicity test under CSCL[2].

1.2. Plan

In order to achieve the purpose, the test design should be considered and followed according to OECD Test Guideline 407[1] or the 28-day repeated-dose toxicity test under CSCL[2].

1.2.1. Selection of Animal Species, Strain and Sex

For the purpose of PCR prediction, only male rats have been confirmed to be used. SD or F344 rats are recommended for PCR prediction. The other conditions (age etc.) should be followed according to the guidelines.

1.2.2. Number of Animals

For the purpose of qPCR prediction, at least three male animals per group should be provided to perform significant difference tests for the gene expression analysis.

1.2.3. Doses

Dose levels should be selected according to the manner of the above mentioned test guidelines.

For the purpose of qPCR prediction, at least two test groups and a vehicle control group should be used.

1.2.4. Administration of doses

The animals should be dosed with the test substance or vehicle for a period of 28 days.

Gavage is recommended since gavage has been confirmed to be used in this qPCR assay. However, administration via diet or drinking water may be used with the same considerations described in the above mentioned test guidelines.

1.2.5. Anesthesia for animal euthanasia

CO_2/O_2 and isoflurane anesthesia are confirmed to be acceptable since these forms of anesthesia have no effects on GEA for qPCR prediction.

1.2.6. Sampling and Storage of Liver

Cautions

mRNA is very unstable and readily degraded by RNA degradation enzyme (RNase) present in the animal sample or released from persons/people. Therefore, liver samples should be handled after avoiding and inactivating RNase with great caution. Specifically;

- ✓ *Designate "RNase-free space",*
- ✓ *Prepare RNase-free materials (tubes, tweezers, pipettes, reagents etc.) that are used for sampling, handling or storage,*
- ✓ *Wear a clean mask,*
- ✓ *Wear gloves,*
- ✓ *The use of RNase away or dry heat sterilization (180 °C, 2-hr) is strongly advised to be ready for sample handling.*

(1) Sampling and Storage of Liver Sample

Animals are euthanized by bleeding from the abdominal aorta CO_2-O_2 (4:1) anesthesia. The liver is immediately excised and weighed. A piece of tissue 2-3 mm wide is taken from the central part of the left lateral lobe of the liver (along the diagonal line connecting the portal vein side and the opposite side).

After confirming that the weight of the tissue is 0.75 g or less, it is immersed in a preservation solution (RNAlater®, Thermo Fisher Scientific) 5 times or more the weight of the sliced sample.

The remaining liver sample can be used for histopathological examination.

(2) Storage until total RNA Extraction

If total RNA is not extracted immediately after sampling, the liver sample should be saved frozen below -80 °C.

2. PREPARATION OF TOTAL RNA

2.1.Extraction of total RNA

2.1.1.Equipment and Reagents

- Precision electronic balance (Mettler Tolede, AB104-S etc.)
- TissueLyser (QIAGEN) or equivalent
- NanoDrop1000 (Thermo Fischer Scientific) or equivalent
- miRNeasy Mini Kit (QIAGEN, Cat. No. 217004) or equivalent
- Chloroform (Wako, 038-02606) or equivalent
- Ethanol (Wako, 057-00456) or equivalent
- Nuclease-free water (Invitrogen, 10977-015) or equivalent

2.1.2. Procedures of Extraction of total RNA

If the sample was stored in RNAlater®, miRAeasy Mini Kit should be used for total RNA extraction.

1) Transfer the frozen RNAlater dipped liver samples at 80ºC to room temperature in order to dissolve the RNAlater solution (about 15 to 30 minutes)
2) Precisely weigh 5 to 30 mg from the portion of the liver sample, place it in a 2 mL tube, add one zirconia beads.
3) Add 750 µL QIAzol and crush the sample with TissueLyser for 5 min at 25 Hz. Furthermore, rotate the interior of the block 180 degrees, attach on TissueLyser to crush the sample at 25 Hz for 5 minutes.
4) After leaving at room temperature for 5 minutes, add 150 µL of chloroform
5) Vortex for 15 seconds or more and leave for 2 minutes
6) Centrifuge at 4 °C, 13,000 rpm for 15 minutes
7) Transfer the supernatant (400 µL) to a new 1.5 mL tube (Caution! Avoid contamination from the precipitation).
8) Add 400 µL of 70% ethanol, pipet well for mixing
9) Transfer 700 µL of mixture to miRNeasy Mini Spin Columns with collection tube attached, put a lid on and centrifuge at 13,000 rpm for 15 seconds to remove waste liquid.
10) Transfer the remaining solution to the same column, centrifuge at 13,000 rpm for 15 seconds, remove the waste liquid.
11) Add 700 µL Buffer RW 1 to the column, centrifuge at 13,000 rpm for 15 seconds, remove the waste liquid.
12) Add 500 µL of Buffer RPE to the column, centrifuge at 13,000 rpm for 15 seconds, remove the waste liquid.

13) Add 500 μL Buffer RPE to the column, centrifuge at 13,000 rpm for 15 seconds, remove the waste liquid.
14) Add 500 μL of 70% ethanol to the column, centrifuge at 13,000 rpm for 15 seconds, remove the waste liquid.
15) Centrifuge at 13,000 rpm for 2 minutes again, then move the column to new 1.5 mL elution tube.
16) Add 30 or 50 μL of RNase free water and leave for 1 minute.
17) Centrifuge at 8,000 rpm for 2 minutes to elute total RNA.

2.2. Concentration Measurement of total RNA and Storage

The absorbance of aliquot of the extracted total RNA was measured by a micro-absorptiometer (NanoDrop 1000 or equivalent) to calculate RNA concentration, A260/280 ratio, and A260/230 ratio.

Only total RNA that fulfill all criteria in Table 1 should be accepted.

Table 1 Criteria-1 for total RNA

Items	Criteria
RNA concentration	100 ng/μL or more
A 260/280 ratio	1.8 or more
A 260/230 ratio	1.5 or more

The aliquot of total RNA solution is dispensed to give 100 or 200 ng/μL of total RNA solution.

The remaining total RNA solution (stock solution) and the prepared 100 or 200 ng/μL total RNA solution are stored at -80 °C.

2.3. Quality Check of total RNA by Bio-analyzer

2.3.1.Equipment and Reagents

- BioAnalyzer 2100 (Agilent Technologies)
- RNA-6000 Nano reagent Kit (Agilent Technologies、PN:5067-1511)
- RNA 6000 Nano LabChip Kit (Agilent Technologies、PN:5067-1511)
- Nuclease-Free Water (Invitrogen、10977-015 or equivalent)

2.3.2. Quality Check of total RNA

RNA 6000 Nano LabChip Kit should be used for sample preparation, and BioAnalyzer 2100 should be used for measurement of RIN (RNA Integrity Number).

1) Aliquot 1 μL from 100 or 200 ng/μL of total RNA solution, and dilute 2-fold with 1 μL of Nuclease-Free Water.
2) Transfer 550 μL of Nano gel matrix to a spin filter and centrifuge at 1,500 g for 10 minutes at room temperature.
3) (preparation of Gel-Dye Mix) Add 1 μL of Nano dye to 65 μL of Nano gel matrix and thoroughly stir.
4) RNA ladder and 2 μL of sample (total RNA solution) are heated at 70 ℃ for 2 minutes to denature, and leave it for 5 minutes on ice.
5) Apply 9 μL of Gel-Dye Mix to the laboratory's ◎ mark (1 place), lower the plunger of the Chip station to the 1 mL scale and keep it for 30 seconds.
6) Remove the plunger and wait for 5 seconds. Then, remove the laboratory chips and apply 9 μL of Gel-Dye Mix to ◎ mark (2 places).
7) Apply 5 μL of Sample buffer to 12 places and  mark (1 place)
8) Apply 1 μL of RNA Ladder (heat denatured) to  mark (1 place)
9) Apply 1 μL RNA sample (heat denatured) to 12 places
10) After mixing the laboratory chips at 2,400 rpm for 1 minute with a dedicated vortex mixer (IKE), attach the laboratory chips to BioAnalyzer 2100 and start the measurement (about 30 minutes).

2.3.3. Judgments

The RIN (RNA Integrity Number) value is used for the quality control measure of total RNA.
RIN value

Calculate the RIN value using the data from BioAnalyzer. If the RIN value is 7.0 or more, the sample can be used for further analysis.

Table 2 Criteria-2 for total RNA

Items	Criteria
RIN value	7.0 or more

3. QUANTITATIVE PCR EXPERIMENT

The method for qPCR should be followed the method described in Saito F et al [4].

3.1. Equipment and Reagents

- ThermoScript III Reverse Transcriptase (Life Technologies、SKU#12236-014)
- DNase/RNase-Free Distilled Water (Invtrogen 10-977-015 or equivalent)
- SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara bio、RR420S)
- Applied Biosystems 7000/7500 Real-time PCR system (Life Technologies) or equivalent
- Heat Block (ASTEC BI-525A) or equivalent

3.2.Procedures of qPCR Experiment

3.2.1. Reverse Transcription Reaction (cDNA Synthesis)

1) Transfer 500 ng to 5 μg mRNA into a 1.5-mL DNase-RNase free tube on-ice and adjusted to 9.0 μL with DNase-RNase free water.
2) Prepare "Primer Mix" by mixing the following reagents and add 3 μL Primer Mix to the 1.5-mL tube.

Primer Mix	1 reaction
Oligo $(dT)_{20}$ Primer	0.5 μL
Random-hexamer Primer or Random primer	0.5 μL
dNTP mix (10 mM)	2.0 μL
	3.0 μL

3) Mix by tapping, heat at 65 ℃ for 5 minutes in a heat block, then cool on-ice for 3 minutes.
4) Prepare “RT master Mix” by mixing the following reagents and add 8 μL of RT Master Mix to the 1.5-mL tube.

RT Master Mix	1 reaction
5×First-Strand Buffer	4.0 μL
0.1M DTT	1.0 μL
RNaseOUT (40 unit/μL)*	1.0 μL
DEPC-treated water	1.0 μL
ThermoScript™ RT (15 units/μL)*	1.0 μL
*Ice-cold storage	8.0 μL

5) After reacting at 25 ℃ for 10 minutes → 55 ℃, 60 minutes → 85 ℃ for 5 minutes in a heat block, cool it for more than 5 minutes with on-ice.

6) Store at -20 ℃ or less until use.

3.2.2. qPCR Reaction

Real-time PCR is performed using the specific primer sequences of four prediction genes (Abcb1b, Eprs, Map3k8 and Igh-6 in Table 3).

Table 3 Gene names and primer sequences used for qPCR

Gene symbol	GeneName	Gene ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
Abcb1b	ATP-binding cassette, subfamily B (MDR/TAP), member 1B	24646	TCATGAGCTGGGA GTATTTGAGG	GTCTCTGAAGG CTTCTCGTCTTG	130
Igh-6	immunoglobulin heavy chain 6	299357	GAATGGAACTCCG GAGAGAC	GTGTGGGTTTA CCAGTGGAC	103
Eprs	glutamyl-prolyl-tRNA synthetase	289352	CCGATTGCCATCCG TCCTA	GCACCGATGGT TGAGCCTGA	102
Map3k8	mitogen-activated protein kinase kinase kinase 8	116596	GCCCAGTCTGATG ACCATGTG	GCAGCGACTCT GAATGTTCCTT	62
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	24383	GGCACAGTCAAGG CTGAGAATG	ATGGTGGTGAA GACGCCAGTA	143

1) The starting amount of cDNA should be adjusted to 50 pg/μL to 5 ng/μL with DNase-RNase free water (Note: In the same liver sample, the starting amount of cDNA should be the same concentration of the prediction genes and an internal standard gene).
2) Add cDNA (1 μL/well) of each liver sample to a 96-well PCR plate
3) Prepare "Q-PCR Master Mix" by mixing the following reagents and add 19 μL Q-PCR Master Mix to each well.

Q-PCR Master Mix	1 reaction
SYBR Premix Ex Taq II (2×)*	10.0 μL
10 μM Forward Primer (final concentration: 0.4 μM)	0.8 μL
10 μM Reverse Primer (final concentration: 0.4 μM)	0.8 μL
ROX Reference Dye (50×)	0.4 μL
ddH_2O	7.0 μL
*Ice-cold storage	19.0 μL

4) React under the following qPCR conditions;

Steps	Temp.	Time	Number of Times
Heat denaturation	95℃	00:30	1 time
qPCR reaction	95℃	00:05	35 times
	50~60℃*	00:34	
Dissociation reaction	95℃	00:15	1 times
	60℃	1:00	
	95℃	00:15	

* Set according to the priming temperature of the primer.

5) [Optional] Further react at 95 ℃→55 ℃ for Melting curve analysis.

6) Using the CT value from qPCR analysis, the gene expression level is calculated relative to the expression level of the internal standard gene Gapdh.

4. PREDICTION OF LIVER CARCINOGENICITY

4.1.Equipment and Software

・Tox-screen (Microsoft Excel (Microsoft))

[Available from http://www.cerij.or.jp/research_assistant_project/tox_omics/37_Tox-screen_Ver1.00.xlsb]

4.2.Data Analysis

The relative differences in the gene expression levels are calculated using threshold cycle (CT) values that were firstly normalized to those of the Gapdh gene as the endogenous control in the same sample. The expression level of each prediction gene for the chemical–treated group should be calculated from the expression level of the same genes from the vehicle control group. The obtained expression level is used for predicting carcinogenic potential by using the criteria in Table 4.

The expression level of any of the prediction genes among Abcb1b, Igh-6 or Eprs is more than 1-fold (log2 scale) in the chemical-treated group higher than that in the vehicle control group, and/or the expression level of Map3K8 was less than -1 fold (log2 scale) of that in the control group, meaning that the prediction result is regarded as carcinogenic in liver. All prediction genes have not met the criteria in Table 4, meaning that it is judged as a non-carcinogenic in liver.

Table 4 Criteria for Carcinogenic Prediction

Genes	Criteria (Expression level)
Abcb1b	2-fold or greater [1-fold or greater (log2 scale)]
Igh-6	
Eprs	
Map3K8	0.5-fold or less [-1 fold or less (log2 scale)]

5.REFERENCES

1) OECD. OECD GUIDELINES FOR THE TESTING OF CHEMICALS (TG 407) Repeated Dose 28-Day Oral Toxicity Study in Rodents. 2008.

2) Ministry of Economy, Trade and Industry. Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc, Chemical Substances Control Law. 2009.

3) Yamashita K, Matsumoto H, Saito F, Takeyoshi M. Differences in gene expression profiles in liver caused by different types of anesthesia: cases of CO_2-O_2 and isoflurane. J Toxicol Sci. 2015;40(6):829-836.

4) Saito F, Matsumoto H, Akahori Y, Takeyoshi M, Simpler alternative to CARCINOscreen® based on quantitative PCR (qPCR). J Toxicol Sci. 2016;41(3):383-390.