Attendance Report

MU Scholarships for Undergraduate Student Exchange Program 2016

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Instructions: This attendance report consists of three parts; 1). General information 2). Background and summary of the exchange activities and 3). Comments and suggestions. Please provide details about your experiences during your exchange program.

PART 1 – General information

1. Name – Surname: Jitrana Kengkanna

2. Gender ☐ Male ☑ Female

2. Faculty / College: Science

3. Type of scholarship

☑ Partial scholarship ☐ Full scholarship

4. Length of exchange program

☑ 30 days (Aug, 17th to Sep, 16th)

☐ 90 days or 1 semester (..... to.....)

5. Host University: Nagoya University

Country: Japan

6. Type of exchange program

☐ Internship – professional experience

☑ Internship – research

☐ Classroom - based

☐ Others, please specify: .....

7. Credit transfer ☐ Yes ☑ No

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PART 2 – Background and summary of the exchange program

Please provide full details about the exchange activities which you have participated in. You should follow the format below when describing the activities. Additional pages are allowed if space is limited.

1. Objectives of the exchange program

To learn the new techniques or methods and use laser Microdissection for studying about the gene expression in maize roots.

2. Exchange activities and output

I did an experiment to study genes that related to arenchyma formation in maize roots by using Laser Microdissection, RNA extraction, and Real-time PCR I made a laboratory report attached in this attendance report.

3. The cultural and academic experiences gained from exchange program

I saw Japanese works and learned their culture, lifestyle, and language. Japanese are diligent, responsible, and kind. They gave me warm welcome and brought me to travel to many beautiful places and eat delicious Japanese foods. For academic experiences, I got many new knowledges. I did the experiment and used high technology devices that I have never used before. They will be useful for my study in the future. This is a valuanable experience for me.

PART 3 – Implications and limitations

What are the implications and limitations which you have encountered during your exchange program? Any comments or suggestions for the improvement of the scholarship scheme?

First, I think language was an important problem that I had encountered during my exchange program because Japanese don't use English in daily life and many things have only Japanese descriptions. I often brought the wrong things when I went to the supermarket However, Japanese were so kind to help and bring me to find the things that I wanted Another problem was money. Japan has higher cost of living than Thailand. I had to save money as much as possible. So I would like to suggest the scholarship scheme to provide more money for students who exchange to the countries that have high living expenses such as Japan, Singapore, Australia etc

Please return the attendance report to International Relations Division within 30 days after the completion of the exchange program.
Email: opinter@mahidol.ac.th Fax: 0 2849 6237

Laboratory Report

Real-time RT-PCR analysis of genes related to aerenchyma formation using laser-microdissected cortical cells of maize roots

By

Jitrana Kengkanna

Department of Biology, Faculty of Science

This report is a partial fulfillment of Mahidol University Undergraduate Exchange Scholarship Program at Nagoya University, Japan from August 17th, 2016 to September 16th, 2016

Abstract

Aerenchyma is an important trait to help plants surviving in many stress conditions including waterlogging. Several studies indicated that prolong period of waterlogging can enhance aerenchyma formation –creating continuous gas space between shoot and root. Ethylene is a key factor to induce program cell death (PCD) in root cortical cells, thereby leading to aerenchyma formation at last. The objective in this study was to investigate whether candidate genes involved in aerenchyma formation are up-regulated in the cortical cells of maize roots in response to waterlogging stress. Maize is a crop that is affected by waterlogging on reducing yields. Therefore, this study used maize roots in aerobic condition and waterlogged condition as the samples that were embedded in paraffin for section and using Laser Microdissection (LM) to collect cortical cells. After that, RNA was extracted from the LM-isolated tissues and then transcript levels of the candidate genes (*ERF2* and *CEL* genes) for aerenchyma formation were analyzed using real-time RT-PCR. The results showed that *ERF2* gene was significantly higher gene expression in waterlogging than in aerobic condition (P=0.04) and *CEL* gene was also significantly greater gene expression in waterlogging than in aerobic condition (P=0.02). In conclusion, waterlogging can induce *ERF2* and *CEL* to be higher expression than aerobic condition. Thus, these genes should be associated with aerenchyma formation because *ERF2* gene is induced by ethylene and then regulates expression of the ethylene responsive genes, which may control aerenchyma formation, and *CEL* gene encodes cellulase enzyme, which is involved in cell wall degradation during aerenchyma formation. These results suggest that *ERF2* and *CEL* genes are involved in induction of aerenchyma formation in response to waterlogging stress.

Introduction

Waterlogging is a worldwide environmental problem, in particularly arid and semi-arid countries (Ojo et al., 2011). Around 10% of irrigated land in the world may suffer from waterlogging. It has meaning that the crop productivity is decreased by waterlogging stress (Muir, 2013). Most of agricultural crops cannot grow well under prolong waterlogged conditions because the soils are filled with water instead air causing the roots lacked oxygen for respiration (FAO, 2001).

Maize is one of the most important crops. It is a staple food for human around the world and animal feeds. Maize production is affected by waterlogging. It is a serious problem for the growth of maize that presented lower leaf portion, leaf senescence and became bronze in color. Moreover, waterlogging is influenced the maize yields (Zubairi et al., 2012).

Plants can respond to waterlogged condition through adaptation and change some characteristics to survive such as aerenchyma formation. Aerenchyma is an important trait to enable and transport many gases to each part of the plants. It was formed by lysis of cortical cells via Programed Cell Death (PCD). Many studies indicated that waterlogging can induce aerenchyma formation and ethylene is one of the important plant hormones to enhance molecular mechanism for promoting aerenchyma formation (Takahashi et al., 2015).

Rajhi et al. (2011) identified many genes that involved lysigenous aerenchyma formation in maize root. There were 575 genes in the cortical cells expressed up-regulated and down-regulated under waterlogging. Those genes related with different expressing in many pathways such as ROS generation/scavenging-related genes, calcium signaling-related genes, cell wall modification-related genes and protein kinase, protein phosphatase, and transcriptional regulator genes.

ERF2 gene and *CEL* gene are candidate genes associated with aerenchyma formation. *ERF2* encodes an AP2 domain-containing transcription factor (i.e. ethylene response factor) and *CEL* encodes endoglucanase (i.e. cellulase), which is involved in cell wall degradation (Rajhi et al., 2011).

In this study, we observed gene expression levels of *ERF2* and *CEL* in maize under aerobic and waterlogged conditions. Cortical cells of root grown in aerobic condition and waterlogged condition were collected by using Laser Microdissection (LM), extracted RNA and then analyzed gene expression using Real-time RT-PCR. Finally, statistical analysis was done for comparing the relative quantities of mRNA between two conditions.

Material and method

Plant material and growth conditions

The samples of maize (*Zea mays* L.) primary roots were fixed and then embedded in paraffin. The length of roots in paraffin was around 0.5 mm. There are 2 conditions, which were aerobic condition, the roots never submerged in distill water and waterlogged condition, the roots were submerged in distill water for 12 hours after planting for 3 days.

Laser Microdissection (LM)

Root samples inside the paraffin were sectioned by using microtome at thickness of 14 μm. Serial sections were placed onto PEN membrane glass slides with RNA secure at 55°C and then using pipette to remove RNA secure and the slides were transferred to the refrigerator for 30 min. After that the slides were dipped in Histoclear to remove paraffin for 10 min twice. Next, the slides were put on tissue paper for air dying and then LM was used to collect cortical cells from the root cross-sections (Rajhi et al., 2011).

RNA extraction and RNA quantities measuring

The cortical cells collected from LM were extracted for RNA by using PicoPure™ RNA isolation kit according to the manufacturer's instructions and Quant-iT™ Ribogreen RNA® Assay kit were used for measuring quantity of extracted total RNA. The instructions were also according to manufacturer (Rajhi et al., 2011).

Real-time RT-PCR analysis

Relative mRNA levels were measured by using Reverse-Transcription Polymerase Chain Reaction (RT–PCR) and StepOnePlus™ real-time PCR system. The fist step of the methods was adding SYBR buffering, Rox reference, RNase Free water, primer (*ZmERF2*, *ZmCEL*, *ZmUBQ*) and reverse transcriptase enzyme to eppendorf tubes. After that, the solutions in eppendorf tubes were added to MicroAmp fast 96-wells Reaction Plate (0.1 mL) in 9 μl each well. Next, standard rRNA, which had concentration at 1000, 100, 10, 1 and water (negative control) were added in 1 μl each well and then qPCR Seal was used for covering the plate. The plate were spin and taken to the Real-time PCR machine. The

conditions of PCR amplification were initial denaturation (95°C for 10 s) and 50 cycles of denaturation (95°C for 5 s), annealing (55°C for 30 s) and extension (95°C for 30 s). Transcript levels of two genes (*ERF2* and *CEL*) were normalized to the transcript levels of the maize Ubiquitin (*ZmUBQ*) gene (Rajhi et al., 2011).

Statistical analysis

Statistical analysis by one-way ANOVA with IBM SPSS Statistics version 24 was used for comparison between growth conditions and relative RNA quantities. The protected LSD posthoc test ($\alpha = 0.05$) was used for multiple comparison tests.

Laser Microdissection

Elements of cortical cells in the root sections were cut by UV laser and then were collected in AdhesiveCap (Figure 1). The number of elements that LM isolated from the cortex was around 200-300 elements (tissues) in each root samples and the total areas of all elements were around 3 million to 4 million μm^2.

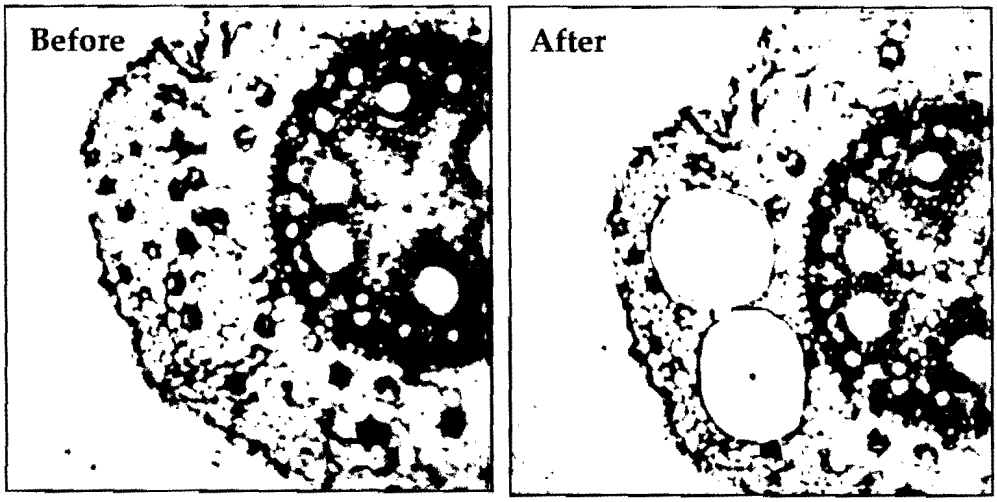

Figure 1 Before and after of using LM isolated cortical cells from maize root cross-section: using laser cut specific-tissue in cortex before (left). UV laser cut selected-tissue already and collected it to AdhesiveCap later (right)

RNA quantities measuring

The quantities of RNA obtained after RNA extraction were 0.61 ng/ml in aerobic1 conditions, 0.81 ng/ml in aerobic2 conditions, and 0.36 ng/ml in waterlogged condition (Table 1). After that, the concentration units were changed to pg/µl and adjusted to be equal concentration in 3 samples at 100 pg/µl and then how many each sample volumes were calculated to use for preparing solution 50 µl as samples for Real-time RT-PCR later.

Table 1 The concentration of RNA in each growth condition.

Growth Conditions	ng/ml	pg/ml	pg/µl (From total volume 800 µl)	Adjust concentration to 100 pg/µl	Volume of samples in solution
Aerobic1	0.61	610	488	0.205	10.246
Aerobic2	0.81	810	648	0.154	7.716
Waterlogging	0.36	360	288	0.347	17.361

Real time RT-PCR of *ERF2* gene

The relative quantity of mRNA from *ERF2* was described in descriptive statistical data (Table 2). Mean of relative mRNA quantities in aerobic1 and aerobic2 conditions were not significant difference, but when comparing between aerobic conditions (1&2) and waterlogged condition, the relative quantity of mRNA was significantly higher in waterlogged conditions than in aerobic conditions (P=0.04) (Figure 2).

Table 2 The descriptive statistical data of *ERF2* gene

Growth Conditions	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Aerobic1	3	.3187771	.13619525	.07863236	-.0195506	.6571049	.17371	.44390
Aerobic2	3	.2749971	.05274710	.03045355	.1439660	.4060282	.21479	.31306
Water-logging	3	.8492021	.19082222	.11017126	.3751734	1.3232307	.64078	1.01532
Total	9	.4809921	.30175895	.10058632	.2490396	.7129446	.17371	1.01532

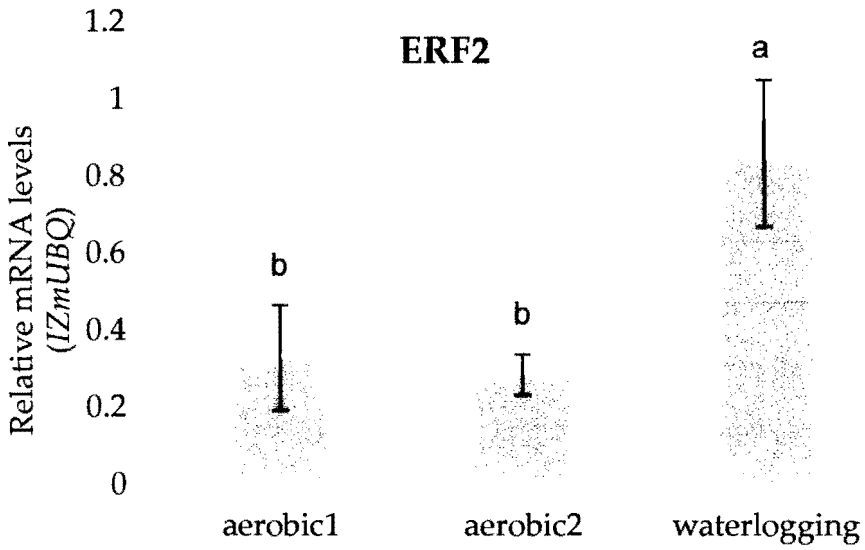

Figure 2 Relative mRNA levels of *ERF2* under waterlogging were significantly greater than under aerobic condition (P<0.05). Data shown are mean of three replicates ± SD. Different letters indicate significant difference.

Real time RT-PCR of *CEL* gene

The relative quantity of mRNA from *CEL* was described in descriptive statistical data (Table 3). This gene had the same result of *ERF2*. That is mean of relative mRNA quantities in aerobic1 and aerobic2 were not significant difference, but relative mRNA quantity was significantly higher under waterlogging than under aerobic1 and aerobic2 conditions (P=0.02) (Figure 3).

Table 3 The descriptive statistical data of *CEL* gene

Growth Conditions	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Aerobic1	3	.2632045	.06405319	.03698113	.1040875	.4223214	.20862	.33372
Aerobic1	3	.3433460	.07085841	.04091012	.1673239	.5193680	.26520	.40341
Water-logging	3	.6124613	.07085860	.04091023	.4364388	.7884838	.53812	.67923
Total	9	.4063372	.16923186	.05641062	.2762541	.5364204	.20862	.67923

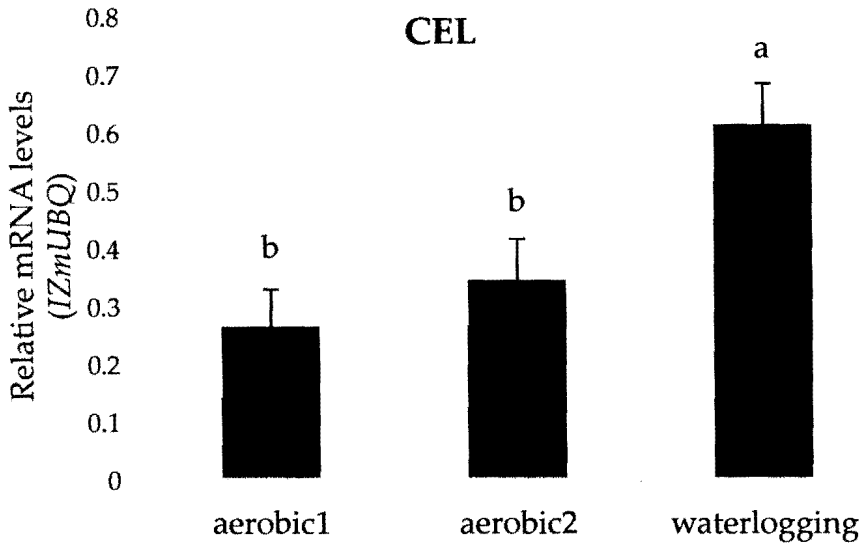

Figure 2 Relative mRNA levels of *CEL* under waterlogging were significantly greater than aerobic condition (P<0.05). Data shown are mean of three replicates ± SD. Different letters indicate significant difference.

Discussion

High amount of RNA was not obtained because the cortical cells were collected from root cross-section using LM. However, as real-time PCR was a sensitive technique of detecting mRNA, transcripts of the two genes were successfully detected in this experiment and can be used for statistical analysis, which indicated the significant difference level of gene expression between aerobic conditions and waterlogged condition.

ERF2 and *CEL* genes had greater relative mRNA quantities in waterlogged condition than in aerobic conditions. This result suggest expression of *ERF2* and *CEL* genes is up-regulated in waterlogged condition and the genes are associated with aerenchyma formation that is also promoted by waterlogging. The *ERF2* gene encodes an ethylene response factor. After maize roots submerged in water for several hours, ethylene is increased in roots. This plant hormone can induce the expression of *ERF2* gene. CEL is involved in cell wall modification or degradation, which is the final step for aerenchyma formation. It is known that expression of the *CEL* gene is regulated through ethylene signaling (Takahashi et al., 2015), suggesting that waterlogging-induced ERF2 protein controls the expression of *CEL* gene, whose products lead to program cell death (i.e. aerenchyma formation) eventually.

Acknowledgements

I am very thankful Professor Mikio Nakazono and Dr. Patompong Saengwilai to give an opportunity for doing this experiment in Japan. I thank Nobusato Mitsuhiro, Watanabe Kohtaro and Gong Fangping for helping and teaching to use Laser Microdissection, RNA extraction and Real-time RT-PCR. Moreover, I thank all members in Nakazono's laboratory for kind support and take care of me. Finally, I thank Mahidol University for support and give the scholarship to learn and get the new experiences.

Reference

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Takahashi H, Yamauchi T, Rajhi I, Nishizawa NK, Nakazono M (2015) Transcript profiles in cortical cells of maize primary root during ethylene-induced lysigenous aerenchyma formation under aerobic conditions. 879–894

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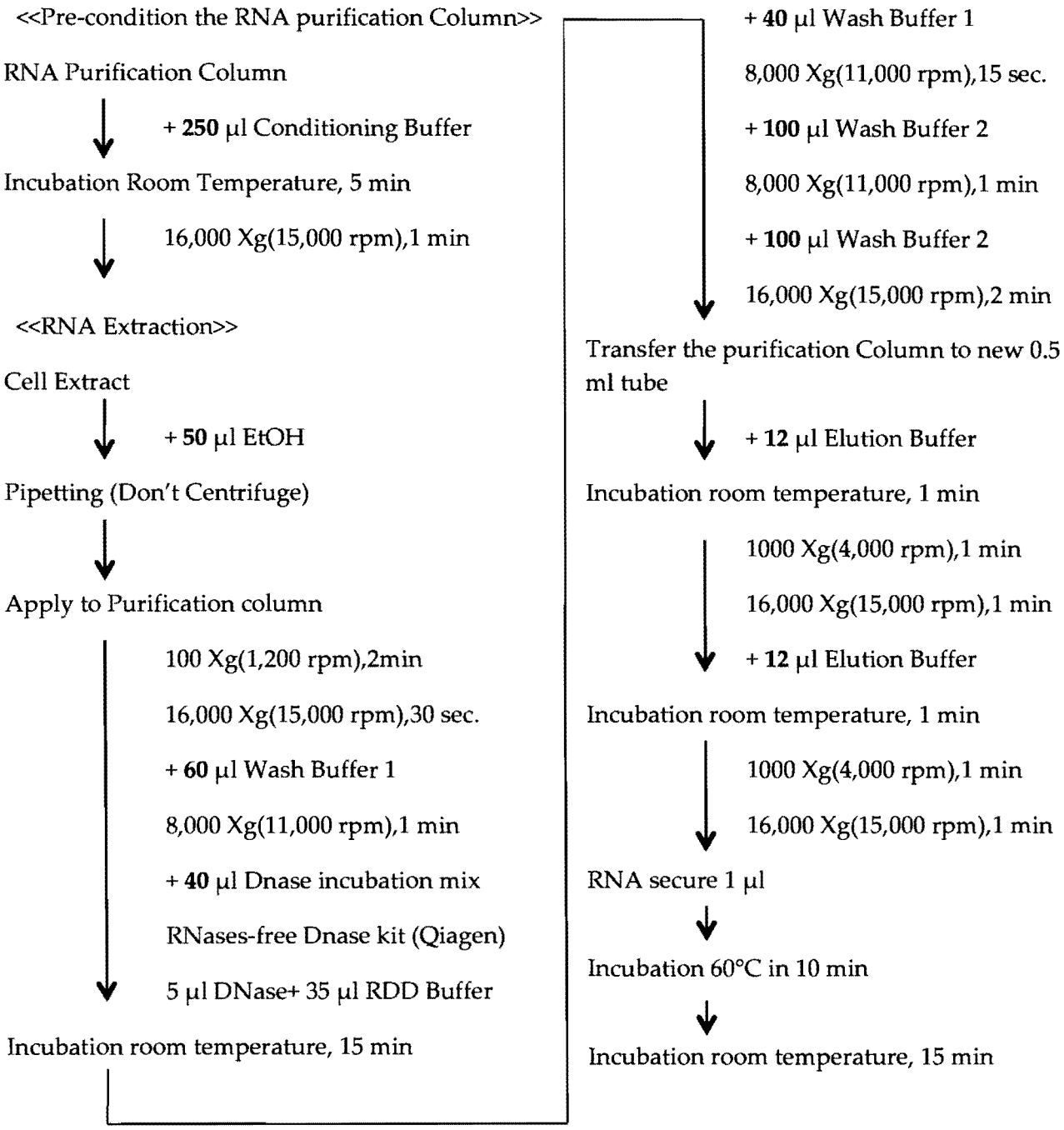
RNA extraction methods

RNA Isolation (PicoPure™ RNA Isolation Kit)



RNA quantities measuring

Quantitative analysis of RNA (Ribogreen)

Preparing 1X TE buffer from 20X TE buffer and 1/2000 Ribogreen from 1X Ribogreen and then adding the solutions followed this table

Final conc. RNA	1X TE Buffer	200 ng/ml rRNA	(1/2000) Ribogreen
0 ng/ml	400 µl	0 µl	40 µl
0.5 ng/ml	398 µl	2 µl	40 µl
1 ng/ml	396 µl	4 µl	40 µl
3 ng/ml	388 µl	12 µl	40 µl
5 ng/ml	380 µl	20 µl	40 µl
Unknown Sample	399 µl	1 µl	40 µl

1.5 ml tube

+ 1X TE Buffer

+ 200 ng/ml rRNA or Sample

+ 400 µl (1/2000) Ribogreen

Incubation Room Temperature, 3 min

Measuring RNA concentration by using spectrophotometer (Ex: 470 nm, Em: 530 nm)