Standard Operating Procedure
for
Hazardous Chemicals

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Building and rooms: Davison Life Sciences Building, Lab B310

Chemical(s)	ABsolute™ QPCR SYBR Green Mix Plus ROX, SuperScript II Reverse Transcriptase, RNase inhibitor, Turbo DNA-*free*™, Agarose, Ethidium bromide (EtBr), Dithiothreitol (DTT).
Process	**SYBR Green QPCR**
Specific Hazards *referred to MSDSs for more detailed information*	EtBr (Ethidium bromide): Potent mutagen, moderately toxic. DTT (Dithiothreitol): may be toxic to central nervous system (CNS). Repeated or prolonged exposure to the substance can produce target organs damage.
Personal protective equipment	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist
Engineering/ventilation controls	Emergency shower and eyewash accessible.
Special handling procedures and storage requirements	Store EtBr away from oxidizers and flammable reagents. Store all stock kits @ -80°C, working kit of SYBR Green Mix Kit @ 4°C, working kit of SSII Reverse Transciptase, RNase inhibitor, and Turbo DNA*free* @ -20°C, all the other buffers on bench top.
Spill and accident procedures *for hazardous chemicals only*	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing and shoes. Rinse for at least 15 minutes. Seek medical attention. Eye exposure: Wash eyes for at least 15 minutes, lifting the upper and lower eyelids. Seek medical attention immediately.
	Small (< 1L): If in solution, absorb freestanding liquid. Use ultraviolet light to locate spill, absorb with spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 1L): Evacuate the room, notify PIs and call 2-5801 to request emergency assistance from Environmental Safety Division
Waste disposal	EtBr gels: gels with <0.1% EtBr can be placed in regular trash; gels with EtBr >0.1% should be collected in a sturdy plastic bag, placed in a cardboard box, labeled and disposed of as non-RCRA hazardous waste via the ESD (Environmental Safety Division).
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: C-J Tsai, 8/31/09
Reviewed by	Name/date: Kate Tay, 8/31/09

SYBR Green QPCR Standard Operating Procedures

Chemicals and Reagents

ABgene ABsolute™ QPCR SYBR Green Mix Plus ROX Vial
Fisher AB 1167A (200 Rxn/Pk, 50µL/Rxn)
Fisher AB 1166B (800 Rxn/Pk, 50µL/Rxn)

QPCR Plastics
Genesee Cat# 24-300, 0.2 ml non-skirted 96-well PCR plate (25/unit)
Genesee Cat# 24-306, 8-well PCR strip flat caps, optically clear (125/unit)
Genesee Cat# 27-125, 0.2 ml 8-well real-time PCR strip tubes, hinged caps (120/unit)

Anchored Oligo dT primer
5'-TTTTTTTTTTTTTTTTTTTTVN (can be custom synthesized)

Random hexamers or decamers (custom synthesized)

SuperScript II Reverse Transcriptase (Invitrogen 18064-022)

RNase inhibitor: 40 U/µL (Ambion 2682)

Turbo DNA-*free*™ Kit (Ambion 1907)

DNase treatment

You should perform the LiCl precipitation step **twice** to avoid co-purifying genomic DNA during RNA extraction. Check RNA quality and assess DNA contamination by electrophoresis or BioAnalyzer. Alternatively, perform a DNase digestion to remove the contaminating DNA, using the Turbo DNA-free kit from Ambion. ***It is important to examine the RNA after DNase treatment to ensure that RNA integrity was not compromised during the procedure.***

Per Ambion, this protocol is designed to remove trace to moderate amounts of contaminating DNA (up to 50 ng DNA/µL RNA, or up to 2 µg of genomic DNA from a 50 µl reaction volume) from purified RNA to a level that is mathematically insignificant in qRT-PCR.

1. Add the following:	Total RNA	1-5 µg	up to 25 µg
	10X Turbo DNase buffer	2.5 µL	5 µL
	Turbo DNase (2 U/µL)	0.5 µL	1 µL
	Nuclease-free water	**to 25 µL**	**to 50 µL**

2. Mix and incubate at 37°C for 30 min. Add 0.1 volume of **resuspended** DNase Inactivation Reagent, mix well and incubate at room temp for 2 min with occasional mixing.

3. Centrifuge at 10,000g for 1.5 min at 4°C to pellet the DNase Inactivation Reagent. Carefully transfer the supernatant (DNA-free RNA) into a fresh tube. ***Avoid disturbing the inactivation reagent pellet because it can sequester divalent cations and interfere with downstream enzymatic reactions.***

4. Per Ambion, the subsequent RT reaction should contain no more than 40% of Turbo DNase-treated RNA in the reaction volume. You may re-precipitate the Turbo DNase-treated RNA to concentrate the sample and avoid interference. Dilute the supernatant by adding 3 volumes of ddH_2O, and precipitate with 2.5 Vol EtOH and 0.1 Vol NaOAc. ***Take Nanodrop measurement and perform QC by agarose gel electrophoresis using 100 ng total RNA (do not skip this important QC step!)***.

RT reaction

Either anchored oligo dT primers or random hexamers/decamers can be used for cDNA synthesis. Oligo dT primers provide better specificity, but may be biased against the 5'-ends. Random primers generate the most diverse cDNA pool with higher yields. For some challenging targets (long or secondary structure-rich), it may be advantageous or necessary to use a mixture of oligo dT and random primers. In our lab, we routinely use the anchored oligo dT primers. Discuss with CJ if you would like to use random primers. **DO NOT CHANGE THE REACTION FORMULA BELOW.**

1. Add the following:

Total RNA	1-5 μg
RNase inhibitor (40 U/μL)	0.5 μL
5X First strand buffer	0.5 μL
0.1 M DTT	0.5 μL
Anchored Oligo dT primers, 100 μM**	0.8 μL
RNase-free water	to 16 μL

** For the dT_{20}-VN primers, 100 μM stock = 664 ng/μL. Invitrogen calls for 500 ng/μL of oligo dT primers, or 250 ng/μL of random primers.

2. Mix and incubate at 65°C for 5 min to denature (secondary structure). Quickly chill on ice and centrifuge briefly at >10,000 rpm.

3. Add the following:

5X First strand buffer	4.5 μL
0.1 M DTT	2.0 μL
RNase inhibitor (40 U/μL)	0.5 μL
10 mM dNTPs	1.0 μL
SuperScript II RT (200 U/μL)	1 μL (total volume = 25 μL)

4. Mix and incubate at room temperature for 10 min and then 42°C for 2 hr.

5. Store cDNA in small aliquots at -20°C until use.

QPCR reaction

Replication: Perform all reactions with 2 technical replicates and 3 biological replicates. ***Technical replicates must be done together in the same plate***

Housekeeping controls: Use 2 housekeeping genes for comparison of target gene expression between treatments (e.g., same tissue type); Use >3 housekeeping genes for gene expression comparison across multiple tissues

Template optimization: Optimum template amount should be determined using 0.1, 0.5, 1, 5, and 10 ng cDNA for each primer pair to confirm linearity of amplification (if samples are limited, use only 0.1, 1 and 10). We found 1 ng (mid-point) to be suitable for most target genes we have tested so far.

Negative controls: For each primer pair and sample type, perform "no-template" and "no-RT" controls to check for non-specific amplification and/or genomic DNA contamination etc.

Primers: Gene-specific primers should target the 3'-UTR regions, or span exon-intron junction, for amplicons of 150-300 bp.

Reaction formula for a single reaction (10 μL reaction volume):

cDNA	0.50 μL (equivalent to 1 ng of total RNA)
2X SYBR master mix	5.00 μL
Forward primer (5 μM)	0.20 μL (equivalent to 100 nM final concentration)
Reverse primer (5 μM)	0.20 μL
Reference ROX dye (1:500)	0.15 μL
ddH_2O	3.95 μL

1. Prepare 3 **master mixes:** (required volume + 10% extra to account for pipetting error)

1.1. Primer mix: (2 μL/rxn)	Forward primer	per rxn: 0.2 μL x ___	= total: _____
	Reverse primer	0.2 μL x ___	_____
	ddH_2O	1.6 μL x ___	_____
1.2. cDNA mix: (2 μL/rxn)	cDNA	per rxn: 0.5 μL x ___	= total: _____
	ddH_2O	1.5 μL x ___	_____
1.3. SYBR mix: (6 μL/rxn)	2X SYBR	per rxn: 5.00 μL x ___	= total: _____
	ROX dye	0.15 μL x ___	_____
	ddH_2O	0.85 μL x ___	_____

2. Add **Primer mix** and **cDNA mix** to each reaction tube/well. Under dim light, add **SYBR mix** to the wells and mix gently.

3. Real-time PCR reaction is performed using the Mx3005P™ Real-Time PCR System (Stratagene) default settings as follows: 15 min at 95°C followed by 40 cycles of 15 sec at 95°C, 1 min at 55-60°C (*depending on primers*) and 1 min at 72°C.