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cDNA Synthesis Using SuperScript III First-Strand Synthesis System for RT-PCR V.3

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[1]Realizing Increased Photosynthetic Efficiency (RIPE)

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protocol .

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Realizing Increased Photosynthetic Efficiency (RIPE)

The SuperScript® III First-Strand Synthesis System for RT-PCR is used to synthesize first-strand cDNA from purified total RNA. RNA targets from 100 bp to >12 kb can be detected with this system.

The procedure follows the manufacturer's instructions but includes lab specific template and reagent amounts and specifies primers and equipment for use in our lab to prepare total RNA samples for qPCR analysis of gene expression.

The original protocol is attached below:
superscriptIIIfirststrand_pps.pdf

Kit: Script™ III First-Strand Synthesis System, ThermoFisher Catalog number: 18080051

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FsuperscriptIIIfirststrand_pps.pdf&title=U3VwZXJTY3JpcHQgSUlJIEZpcnN0LVN0cmFuZCBTeW50aGVzaXMgU3lzdGVtIGZvciI

Lynn Doran 2022. cDNA Synthesis Using SuperScript III First-Strand Synthesis System for RT-PCR. **protocols.io** https://protocols.io/view/cdna-synthesis-using-superscript-iii-first-strand-b64vrgw6 Lynn Doran

protocol

Invitrogen. "SuperScript III First-Strand Synthesis System for RT-PCR." Jan. 2013. Doc. Part No: 18080051.pps, Pub. No.: MAN0001346, Rev. 3.0. Life Technologies. Lab protocol.

There was an inaccurate 65C 5 minute incubation between step 12 and 13 that was removed. Note about random hexamer vs oligo dt primers was included.

cDNA synthesis, RT-PCR Sample Prep

protocol ,

https://www.thermofisher.com/order/catalog/product/18080051#/18080051, Invitrogen by Life Technologies.

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- Always wear gloves and change them often to avoid RNases.
- Use RNase-free solutions.
- Use RNase-free certified, disposable plasticware and filter tips whenever possible.

- Ice
- Dry Ice
- Ice Bucket
- Script™ III First-Strand Synthesis System, ThermoFisher Catalog number: 18080051
- 0.2ml PCR 8-Tube 125 strips flat cap, USA Scientific, 1402-2500
- Pipette, 1-10 ul, Single Channel, Variable, Eppendorf Research Plus
- Tips, pipette, 1-10 ul, TIPONE® FILTER TIP REFILLS Item #1121-2710
- Pipette, 10-100 ul, Single Channel, Variable, Eppendorf Research Plus
- Tips, pipette, 20- 200 ul, TIPONE® FILTER TIP REFILLS Item #1120-8710
- RNase Away, spray, ThermoScientific 21-402-178
- 70% Ethanol
- Thermal cycler, Bio-Rad T100 Thermal Cycler or equivalent
- Mini-centrifuge, Thermo Scientific™ mySPIN™ 6 Mini Centrifuge

RNA extracted from Qiagen RNeasy Plant Extraction Kit, or equivalent quantity and quality.
RNA used for cDNA synthesis for qPCR should be high quality.

- A_{260}/A_{280} values > 1.8, ideally values approach 2.1 (note in SYBR green guide they recommend using samples with >2).
- There is no defined rule for an acceptable A260/A230 ratio for qPCR. A low value is often the result of guanidium thiocyanate contamination from the extraction procedure, and that concentrations of up to 100 mM are tolerated. A good rule of thumb, A260/A230 values should be >1.8, and for pure RNA expect values 2.1-2.3.
- RNA integrity as indicated by Qubit RNA IQ values >5, ideally > 8.

1 Allow reagents to thaw completely, mix, and briefly minicentrifuge 10 mM dNTP mix and 50 ng/ul random hexamers before use. Store on ice when not in use.

2 Label two PCR tubes per sample, RT and NRT.

Best practice for qPCR is to prepare a no reverse transcriptase (NRT) reaction for each of your samples to ensure that minimal genomic DNA remains in your reverse transcriptase sample and that only cDNA, not gDNA, is being interpreted as gene expression in your qPCR results.

3 Treat gloves and pipettes with RNase away and sterilize work area with 70% ethanol before pipetting reagents.

4 If performing many reactions, make a master mix of primer random hexamers, dNTP mix, and water, multiply each component by the number of reactions needed plus at least 20% to account for pipetting error. Remember that you will need two reactions for each sample. Pipette **5 µL** of the prepared master mix into each sample tube, both RT and NRT.

If only a few reactions are needed, pipette the following for 1 reaction into each tube.

A	B	C
Component	**Amount for 1 rxn**	**Amount for 10 rxn**
50 ng/ul Random Hexamers	1 ul	10 ul
10 mM dNTP Mix	1 ul	10 ul
DEPC-treated or MilliQ (18.2 MΩ.cm) water	3 ul	30 ul

The Superscript III kit recommends oligo DT for RT-qPCR targets because it more specifically amplifies the mRNA reducing the complexity of the cDNA product and produces a more consistent end product.

Random Hexamers is used to avoid selection bias against long reads or primers located in the 5' end.

5 Add **5 µL** of high quality RNA from each sample to the respective RT and NRT tubes.

Keep RNA samples on dry ice when not actively in use to inhibit RNases. Return samples to **-80 °C** as soon as possible.

6 Incubate the tube at **65 °C** for **00:05:00** in the thermocycler. 5m

7 Place **On ice** for a minimum of **00:01:00** . 1m

8 Allow reagents to thaw completely, mix, and briefly centrifuge 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U/ul RNase OUT, and 200 U/ul SuperScript III RT before use. Store **On ice** when not in use.

9 Prepare a cDNA Synthesis (RT) Master Mix adding each component in the indicated order. Adjust the table for the number of RT samples.

A	B	C	D
Order	Component	Amount for 1 rxn	Amount for 10 rxn
1	10X RT buffer	2 ul	20 ul
2	25 mM MgCl2	4 ul	40 ul
3	0.1 M DTT	2 ul	20 ul
4	40 U/ul RNase OUT	1 ul	10 ul
5	200 U/ul SuperScript III RT	1 ul	10 ul

10 Add **10 µL** of the cDNA Synthesis (RT) Master Mix to each tube labeled RT.

11 Prepare a cDNA Synthesis (NRT) Master Mix adding each component in the indicated order. Adjust the table for the number of NRT samples.

A	B	C	D
Order	Component	Amount for 1 rxn	Amount for 10 rxn
1	10X RT buffer	2 ul	20 ul
2	25 mM MgCl2	4 ul	40 ul
3	0.1 M DTT	2 ul	20 ul
4	40 U/ul RNase OUT	1 ul	10 ul
5	DEPC-treated or MilliQ (18.2 MΩ.cm) water	1 ul	10 ul

12 Add **10 µL** of the cDNA Synthesis (NRT) Master Mix to each tube labeled NRT.

13 Program a thermocycler for the following and run all samples, both RT and NRT:

A	B	C
Step	Time (m:s)	Temp (C)
1	10:00	25
2	50:00	50
3	5:00	85
4	Infinity	4

The samples can be removed after the 5 minute incubation at 85^{o}C is complete, they do not need to reach 4^{o}C in the thermocycler.

14 Chill **On ice** until samples are cool to the touch.

15 Briefly minicentrifuge all samples.

16 Add **1 µL** of RNase H to each tube (both RT and NRT) and incubate in a thermocycler for **00:20:00** at **37 °C** 20m

17 cDNA samples can be stored at **-20 °C** .