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qPCR Protocol

CELLINK Bioink

This is a suggested procedure, please adjust according to your experimental needs.

Protocol aim

The aim of this protocol is to provide instructions for extracting Total RNA to perform qPCR on 3D bioprinted constructs. This protocol was optimized for CELLINK® Bioink printed with cells, together with TATAA Biocenter. Changing the parameters in the protocol might change incubation time, reagents or needed optimization.

Material needed

RNA Extraction

- 3D bioprinted constructs of CELLINK® Bioink
- 2 ml Eppendorf tubes
- Total RNA Purification Kit #17200 (Norgen Biotek Corp.)
- 96-100% ethanol
- β-mercaptoethanol
- Steel beads
- TissueLyzer (Qiagen, GmbH)
- RNase/DNase free pipette tips
- RNase-free microcentrifuge tube
- 70% ethanol
- RNase-Free DNase I Kit #25710 (Norgen Biotek Corp.)
- NanoDrop (ND-1000 Thermo Fisher scientific)
- Capillary Electrophoresis (Fragment analyzer, Advanced Analyticals Inc)
- Standard Sensitivity RNA Analysis Kit # DNF-471-0500

Reverse transcription

- RNA extraction samples
- TATAA Grandscript™ cDNA synthesis kit #A103
- 96 well plate
- Adhesive clear film
- CFX96 PCR touch (Bio-Rad Laboratories, Inc)

qPCR

- TATAA Probe GrandMaster® Mix #TA02 (TATAA Biocenter AB)
- Adhesive clear film
- RNase free water
- qPCR plate
- CFX96 PCR touch (Bio-Rad Laboratories, Inc)

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Protocol

This protocol can be performed under the fume hood and at room temperature. The protocol includes the three steps, RNA extraction, Reverse transcription and qPCR.
Note: Wipe surfaces and equipment with RNase Away before start of experiment.
Note: All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~14,000 RPM) except where noted otherwise.

RNA Extraction Protocol

Step	Title	Material	Description
1	Prepare RNA sample	- 3D bioprinted construct of CELLINK® Bioink with cells - 2 ml Eppendorf tubes	- Collect the samples in 2 ml Eppendorf tubes and freeze them immediately in the liquid nitrogen or on dry ice before use. - If the plan is to use directly after collection no freezing is required.
2	Reagent preparation (using Total RNA Purification Kit #17200)	- Wash solution A - Buffer RL - 96-100% ethanol - β-mercaptoethanol	- For every new kit prepare a working concentration of the Wash Solution A by adding 90 ml of 96-100% ethanol to the supplied bottle containing the concentrated Wash Solution A. This will give a final volume of 128 ml. The label on the bottle has a box that may be checked to indicate that the ethanol has been added. - In a fume hood, add 10 µl of β-mercaptoethanol to each 1 ml of Buffer RL required. Note: β-mercaptoethanol is toxic and should be dispensed in a fume hood.
3	Lysate preparation and homogenization	- Steel beads - TissueLyzer - RNase/DNase free pipette tips - RNase-free microcentrifuge tube - 70% ethanol	- Add 600 µl Buffer RL mixed with β-MEOH + one steel bead to every sample - Homogenize in Tissuelyzer for 2 min at 25 Hz. Rotate the rack and homogenize for 2 more minutes. - Using RNase/DNase free pipette tips, transfer the lysate into an RNase-free microcentrifuge tube. - Spin the lysate for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate.

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			- Add an equal volume of 70% ethanol to the lysate volume collected (100 µl of ethanol is added to every 100 µl of lysate). Vortex to mix.
4	RNA Binding to Column	- Column - Collection Tube (2 ml)	- Assemble a column with one of the provided collection tubes. - Apply up to 600 µl of the lysate with the ethanol onto the column and centrifuge for 1 minute at ≥ 3,500 x g (~6,000 RPM). Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute at 14,000 x g (~14,000 RPM). - Discard the flow through. Reassemble the spin column with its collection tube.
5	DNase Treatment (Norgen's RNase-Free DNase I Kit)	- Wash Solution A - DNase I - Enzyme Incubation Buffer A	- Apply 400 µl of Wash Solution A to the column and centrifuge for 2 minutes. Discard the flow through. Reassemble the spin column with its collection tube. -For every column reaction to be performed, prepare a mix of 15 µl of DNase I and 100 µl of Enzyme Incubation Buffer A using Norgen's RNase-Free DNase I Kit (Product # 25710). Mix gently by inverting the tube a few times. DO NOT VORTEX. Apply 100 µl of the RNase-free DNase I solution to the column and centrifuge at 1 minute. Note: Ensure that the entire DNase I solution passes through the column. If needed, centrifuge for an additional minute. - After the centrifugation, pipette the flow through that is present in the collection tube back onto the top of the column. - Incubate the column assembly at 25 - 30°C for 15 minutes.

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6	Column Wash	- Wash Solution A	- Apply 400 µl of Wash Solution A to the column and centrifuge for 1 minutes. Discard the flow through. Reassemble the spin column with its collection tube. - Wash the column a third time by adding another 400 µl of Wash Solution A and centrifuging for 1 minute - Discard the flow through and reassemble the spin column with its collection tube. - Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.
7	RNA Elution	- Elution tube (1.7 ml) - Elution Solution A	- Place the column into a fresh 1.7 ml Elution tube provided with the kit. - Add 50 µL of Elution Solution A to the column. - Centrifuge for 2 minutes at 200 x *g* (~2,000 RPM), followed by 1 minute at 14,000 x *g* (~14,000 RPM). If the entire 50 µl has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for 1 additional minute.
8	Concentration Measurement	-Nanodrop spectrophotometer -RNase/DNase free water or Elution Buffer	-Start the nanodrop instrument. -Start the software by clicking the Nano1000 -Choose the Nucleic acid measurement program. -Raise the sampling arm and pipette RNase/DNase free water to clean the measurement pedestal. Wipe away using a dry, lint-free laboratory wipe. -Pipette Elution buffer to blank the instrument. Press blank and then wipe away using a dry, lint-free laboratory wipe. -Pipette the sample onto the lower measurement pedestal.

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			-Lower the sampling arm and initiate a spectral measurement using the software on the PC. -When the measurement is complete, raise the sampling arm and wipe the sample from both the upper and lower pedestals using a dry, lint-free laboratory wipe. - After finishing all the sample concentration measurement, clean the pedestals by measuring RNase/DNase free water two times. -Write down all the concentration or print it.
9	Fragment analysis to checked for RNA Integrity	-Fragment Analyzer (CapillaryElectrophoresis) -Standard Sensivity RNA Analysis Kit	-To check the RNA integrity and the quality. See separate protocol.
10	Storage		- The purified RNA sample may be stored at –20°C for a few days. (It is recommended that samples be placed at –80°C for long term storage).

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Reverse Transcription

Step	Title	Material	Description
1	Samples Preparation	- RNA extraction samples - RNase/DNase free water - RNase/DNase free pipette tips	- Take out the samples from the -80°C freezer and place at 4°C before use. - Normalize the sample with the lowest sample concentration or dilute the total RNA samples at least 1:1 to avoid potential interference with the sample matrix (up to 250 ng total RNA per 10 µl cDNA synthesis reaction has been tested without trace of inhibition).
2	cDNA synthesis	- cDNA synthesis mix - 96 well plate - Adhesive clear films - CFX96 PCR touch	- Prepare the cDNA synthesis mix (see Table 1 below) and store at 4°C before use. - Transfer 2.5 µl of cDNA synthesis mix in each well of a 96 well plate and add 7.5 µl of diluted samples in their respective wells. - Seal the plate with Adhesive clear films. - Vortex the plate followed by spinning at 3000 rpm for 1 min. - Put the plate in CFX96 (Bio-Rad Laboratories, Inc), fill in the cDNA synthesis temperature program (see Table 2 below) and start the run.

Table 1. Mastermix protocol for one cDNA synthesis reaction (normalized samples).

Reagents	Stock conc.	Volume	Final conc.
TATAA GrandScript™ reaction Mix	5X	2 µl	1X
TATAA GrandScript™ enzyme	20X	0.5 µl	1X
Template RNA	-	7.5 µl	-
Total reaction volume:		**10 µl**	

Table 2. Temperature program for cDNA synthesis.

Step	Time	Temperature	Cycles
Activation	5 min	22°C	1
RT	30 min	42°C	1
Inactivation of enzyme	5 min	85°C	1
	∞	4°C (hold)	

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qPCR

Step	Title	Material	Description
1	Prepare Mastermix	-TATAA Probe GrandMaster® Mix -Fwd & Rev Primer -Probe -RNase free water -qPCR plate	- Prepare Mastermix for qPCR (see Table 3). - Pipette the Mastermix to a plate (96-well or 384-well depending on amount of reactions). - Pipette all samples including a No-Template control in desired number of replicates into a plate compatible with the qPCR instrument (96-well or 384-well depending on amount of reactions).
2	Mixing the sample to the Mastermix	- Adhesive clear film - CFX96 or 384 PCR touch	- Seal the plate with adhesive clear film. - Vortex the plate followed by spinning at 3000 rpm for 1 min. - Set the qPCR temperature program on CFX96 or CFX384, see Table 4. Make sure that detection is performed in relevant fluorescence channel(s).
3	Run & Analysis		- Put the plate in CFX96 or CFX384 and start the analysis. Note: For our CELLINK® Bioink, the recommendation is to use GADPH and GUSB gene as a reference housekeeping gene based on normalized expression of genes from TATAA human Reference gene panel. The reference gene screening was performed in GenEx (MultiD analyses AB) and identified GAPDH and GUSB as the two best reference genes.

Table 3. Mastermix protocol for qPCR.

Reagents	Stock conc.	Volume 1 rxn	Final conc.
Primers	10 µM	0.4 µl	400 nM*
Probe	10 µM	0.2 µl	200 nM*
TATAA Probe GrandMaster® Mix	2X	5 µl	1X
RNase free water	-	2.4 µl	-

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Template**	-	2 µl	-
Total reaction volume:		**10 µl**	

* The final concentrations of primers and probe are assay dependent.
**The volume of added cDNA (generated with TATAA Grandscript cDNA synthesis kit) should not exceed 1/5 of the total reaction volume.

Table 4. Temperature program for qPCR.

Step	Time	Temperature	Cycles
Activation	60 s	95°C	1
Amplification	5 s	95°C	Cycle with measurement 45 times
Fluorescence measurement	30 s	60°C***	

***The annealing temperature is assay dependent, but many assays are designed to have optimal performance at 60°C.

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