Direct cDNA synthesis and pre-amplifcation of single embryos for RT-PCR

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Introduction

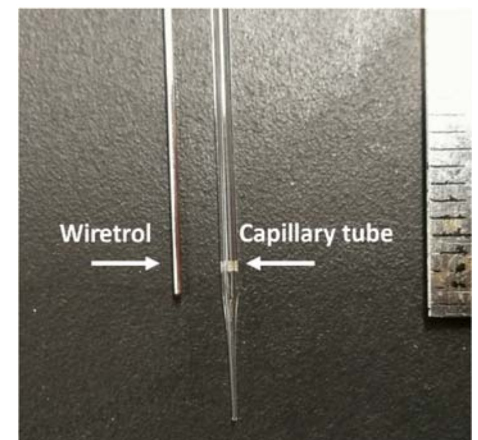
This protocol allows gene expression analysis within single embryos on multiple targets in either conventional real-time PCR or using the Fluidigm platform. The protocol makes extensive use of the CellsDirect Kit from ThermoFisher Scientific (catalog number 11753). References to the kit refer to this product. This protocol describes a method that converts RNA directly into cDNA without RNA extraction, which avoids the variation due to extraction process. The number of genes of interest can be up to more than 100. However, the target genes for analysis have to be determined prior to cDNA synthesis. Once the cDNA is made, there is no way to add additional gene to analyze. The best approach to primer design is to have primers span an intron in the target region. This is not a requirement, however, because of the DNase treatment included in the protocol.

Work flow	Time
Snap freezing*	0.5 ~ 2 h
Cell lysis	0.5 ~ 1 h
DNase treatment	0.5 ~ 1 h
cDNA synthesis and pre-amplification	2 h
Exo I treatment*	1 h

* Optional stop point

Embryo collection and storage

1. Prepare capillary tube for embryo collection: place a capillary tube (5-000-1005, Drummond) in the flame of an alcohol or Bunsen burner. Heat until the glass becomes soft and then pull. Use a mini grinding disc to cut the tube where the outer diameter is around 300 μm. Use this capillary tube for transferring embryos into collection tubes to minimize the amount of solution that introduced.



2. Prepare collection tubes: add 1 μl of resuspension buffer (provided in the kit) into each RNAase-free PCR tube.
3. Wash embryos with DEPC-treated Dulbecco's phosphate buffered saline containing 0.2% (w/v) polyvinylpyrrolidone (DPBS-PVP) three times.
4. Remove zona with Acid Tyrode's (T1788, Sigma).

5. Repeat step 3.
6. Collect each embryo into collection tubes using a pulled glass capillary tube prepared above to avoid introducing excessive solution that dilutes the buffer. Snap freeze in liquid nitrogen.
7. Store each embryo at -80°C.

Cell lysis and DNase I treatment

1. Withdraw samples from -80ºC freezer, place on ice and add 0.5 µl of lysis enhancer (from kit) into each tube under a stereomicroscope. Make sure the embryo does not stick to the pipette tip.
2. Lyse embryos at 70ºC for 20 min in a PCR machine. After 10 min, pause the program, mix each sample by tapping the end of tube and resume incubation.
3. Add 0.5 µl of DNase I (1 U/µl, 1 U digests 1 µg DNA at 37ºC) and 0.22 µl of DNase I buffer from the kit. Mix by pipetting up and down.
4. Digest at 25ºC for 15 min in a PCR machine.
5. Add 0.55 µl of 25 mM EDTA from the kit. Vortex briefly to mix and spin down on a microcentrifuge.
6. Deactivate DNase I at 70ºC for 10 min in a PCR machine. At this point, the volume in tube should be about 2.75 µl.

cDNA synthesis and pre-amplification

1. Prepare primer mix containing 500 nM of each primer. For example, if primers are provided at 100 µM, do a two-step dilution. First, prepare a 10 µM mix by adding 10 µl 100 µM forward + 10 µl 100 µM reverse + 80 µl nuclease-free water. Secondly, add 5 µl 10 µM gene A primer + 5 µl 10 µM gene B primer + 5 µl 10 µM gene C primer +.... + nuclease-free water to a final volume of 100 µl. If primers of more than 20 genes are being mixed together, the second step should involve mixing 0.5 µl of each 100 µM stock instead and bring the final volume to 100 µl. To prepare a 96 primer mix designed for Deltagene assay and provided at 100 µM by Fluidigm, add 1 µl of each primer well into 104 µl of nuclease- free water to create 200 µl of primer mix.

2. Prepare master mix as follows and add 7.5 µl into each tube:

Reagent	Volume per reaction (µl)
CellsDirect 2X reaction mix (from kit)	5
SuperScript III RT/ Platinum Taq mix (from kit)	0.5
Primer mix (500 nM)	1
DNA suspension buffer (T0220, Teknova)	1
TOTAL	7.5

3. Run the following PCR program in a PCR machine:
    1) 50°C, 20 min (for RT)
    2) 95°C, 2 min
    3) 95°C, 15 s + 60°C, 4 min: for 18 cycles.

Exonuclease I treatment to remove primers

1. Using Exonulcease I and the buffer provided by New England Biologicals (M0293S), prepare a master mix (per sample) of 0.8 µl 20U/µl ExoI + 0.4 µl buffer + 2.8 µl nuclease-free water.
2. Run a thermal program on a PCR machine: 37°C, 30 min + 80°C 15 min + 4°C infinity
3. The volume of cDNA in tube is 14 µl. Dilute cDNA 1:5 by adding 56 µl of nuclease-free water and store at -20°C. Assuming PCR requires 1.2 µl cDNA in duplicate, 70 µl should be sufficient to analyze 28 genes by conventional real-time PCR and 96 genes in a Fluidigm run. If needed, the cDNA can be diluted up to 1:10 .

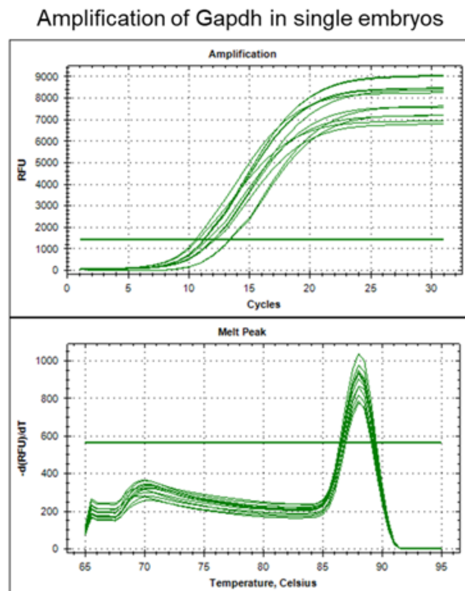
Use of Pre-amplified cDNA in standard RT-PCR

1. Prepare a SsoFast EvaGreen master mix:
   10.4µl EvaGreen (1725211, Biorad) + 1.26 µl of 10 µM forward and reverse primer + 7.1 µl of H_2O + 1.2 µl cDNA. Use 1.2 µl RNase/DNase free H_2O as no template control.

2. Run the real-time PCR program, for example:
    (1) 95°C, 30 s
    (2) 95°C, 5 s
    (3) 60°C, 5 s, + plate read

(4) Go to step (2), 30 more times

(5) Melt Curve, 65 to 95°C, increment 0.5^{o}C, for 5 s, + Plate read



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