

Biological Systems and Methods for Studying WT1 in the Epicardium

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Abstract

The embryonic epicardium is an important source of cardiovascular precursor cells and paracrine factors required for adequate heart formation. During embryonic heart formation, WT1 is mainly expressed in epicardial cells and epicardial derived cells. Its expression has been used to trace epicardial derivatives in embryos and recently it has been used to follow the reactivation of epicardial cells after myocardial infarction. Interestingly, the highest level of expression of WT1 during epicardium development correlates with the highest proliferative state, stem cell properties, and migratory capacity of epicardial cells. Here, we review the various types of tools and strategies used to study WT1 function in the embryonic epicardium and provide examples of their use.

Key words Wt1, Epicardium, Development, Cell culture, In vivo FACS analysis, Migration assay, Transwell assay, Quantitative PCR

1 Introduction

The epicardium originates from a mass of mesothelial cells known as the proepicardium, which is located on the wall of the embryonic pericardial cavity just dorsal and caudal to the developing early heart [1]. Proepicardial cells extend out to reach the surface of the heart around stage E9.5 in mice and spread and cover all the surface of the myocardium by E11.5 [1].

The models in which the epicardium is disrupted are lethal before the onset of normal coronary development and blood flow, suggesting an important role for the epicardium in heart development [2].

The epicardium contributes to the formation of cardiovascular precursor cells that differentiate into various cell types, including coronary smooth muscle and endothelial cells, perivascular and cardiac interstitial fibroblasts, and a low percentage of cardiomyocytes [2]. In addition to cellular contributions to the developing heart, the epicardium and its derivatives provide paracrine signals which influence myocardial maturation and the development of the coronary vasculature [2, 3].

The understanding of the epicardial potential in development is highly relevant given the crucial role of the epicardium in response to injury [4, 5]. Following myocardial infarction (MI), epicardial cells revert to an embryonic-like phenotype, proliferating at the site of injury and secreting factors to modulate wound healing [4, 5].

During heart development, *Wt1* is mainly expressed in the epicardium and epicardial derived cells, *Wt1* being one of the main hallmarks of the embryonic epicardium signature [6]. Its expression is downregulated during the course of epicardium development and interestingly its expression is reactivated after MI [4, 5].

One of the major challenges in epicardial biology is the identification of novel downstream targets of *Wt1* during heart development. Targeted inactivation of the *Wt1* gene causes embryonic lethality at E13.5 due to defects in heart formation [6]. Histological analyses of hearts from *Wt1* null mice clearly demonstrated severe defects in epicardium formation, with some regions of the heart containing gaps and others with a complete absence of an epicardium [6]. A significant number of apoptotic cells have also been identified in the epicardium and subepicardial region of *Wt1KO* embryos [6]. Thus, these KO mice constitute an important limitation for the study of the function of such an important gene for the epicardium beyond stage E13.5 and which might have a crucial role in heart repair.

Recently, we undertook a conditional gene inactivation strategy using a Cre/loxP system to inactivate *Wt1* in the epicardium from mice containing a floxed *Wt1* allele [3]. *Wt1loxP/loxP* mice were interbred with *Gata5-Cre/Wt1GFP+/-* transgenic mice. The resulting *Wt1KO* mice (*Gata5-Cre+; Wt1loxP/gfp*), henceforth known as epi*Wt1KO*, have its *Wt1* conditional allele located opposite the *Wt1GFPKI* allele (*Wt1loxP/gfp*), allowing us the isolation by FACS of control and *Wt1KO* epicardial cells [3]. The resulting *Wt1* mutants die between embryonic days E16.5 and E18.5 due to cardiovascular failure. The good integrity of epicardial cells covering the surface of the myocardium in these KO mice demonstrated the importance of this *Wt1KO* mouse model in the study of *Wt1* function in the embryonic epicardium [3].

However, the fact that epicardial cells constitute less than 10% of the cells of the developing heart complicates epicardium research and renders the study of the function of *Wt1* in epicardial cells difficult. To bypass this problem and to further study the mechanism of action and role of *Wt1* at a cellular and molecular level, we generated a series of immortalized epicardial cell lines.

Firstly, we generated immortalized epicardial cells from control (*Wt1gfp/+*) and KO embryos (*Wt1gfp/-*), where both are GFP positive and express epicardial cell markers; however their morphology is different [7] (Fig. 1). Interestingly, this change in morphology (size) is also observed in the *Wt1KO* epicardium in vivo

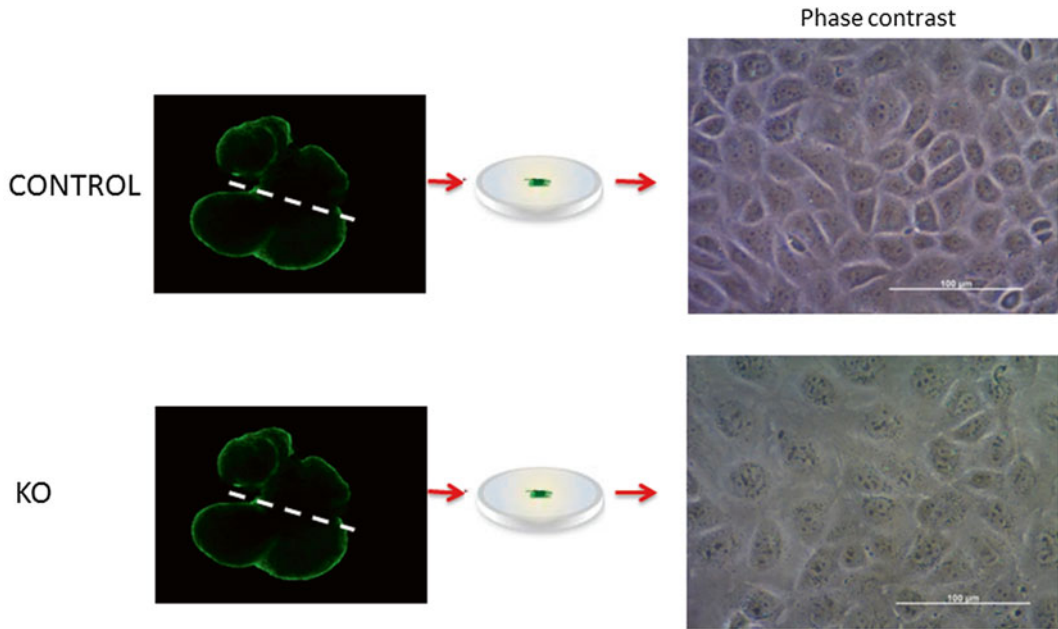


Fig. 1 Generation of immortalized epicardial cells. Schematic representation of the isolation of *Wt1GFP+* E11.5 ventricles (*left panels*) and generation of immortalized epicardial cells from control and *Wt1KO* mice ventricles that were placed on a gelatin coated dish. Phase-contrast micrograph of Control and *Wt1KO* immortalized epicardial cells is shown on the *right hand side panels* where the typical cobblestone morphology of epicardial cells can be observed in both cultures; however KO cells can also be observed to be bigger in size

(data not shown), suggesting that *Wt1KO* immortalized cells and the *Wt1KO* epicardium have acquired some modification during embryonic epicardium development.

We also generated tamoxifen inducible *Wt1KO* epicardial cells [3, 8]. The use of tamoxifen inducible *Wt1KO* cells has helped us in the identification of direct targets of WT1. The genes that are switched on or off shortly after the deletion of *Wt1* are more likely to be directly regulated by WT1.

We generated these immortalized cells using two strategies: the first one involves immortalized epicardial cells that were produced from *Wt1loxP/loxP* mice, and which were transfected with a CAGGs-CreERT2-IRES-puro R expression construct to enable the generation of stable clones using puromycin selection (kind gift from Dr. Lars Grotewold) [3]. The second strategy consisted in the generation of immortalized epicardial cells from CAGG-CreER⁺; *Wt1loxP/gfp* and CAGG-CreER⁻; *Wt1loxP/gfp* mice [8].

These strategies allowed us to generate clones and mixed populations of immortalized epicardial cells; thus we are able to produce enough cells and material to enable us to study the epicardium from a cellular and molecular biology point of view (Fig. 2).

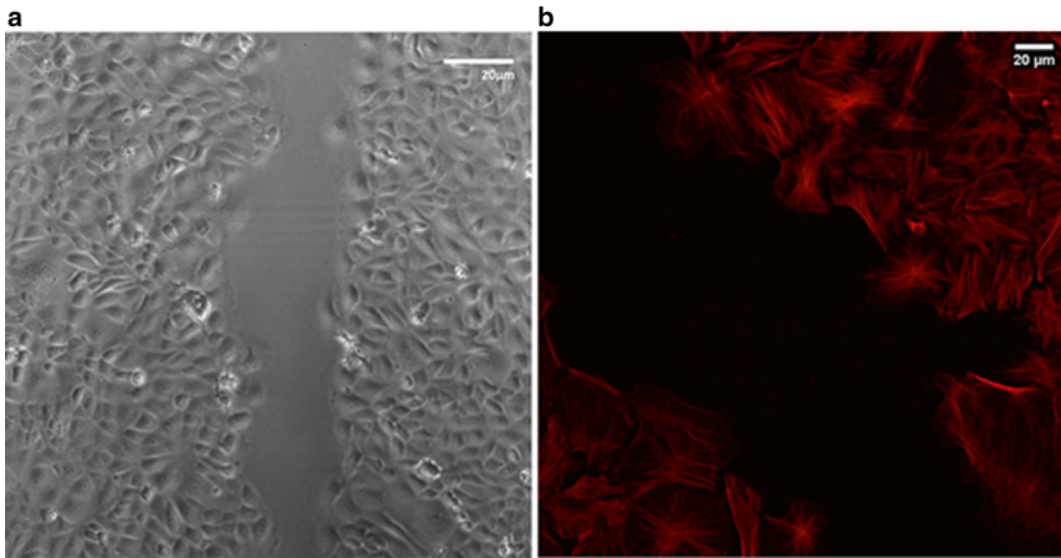


Fig. 2 Migrating epicardial cells in a wound healing assay. **(a)** Phase-contrast micrograph of migrating immortalized epicardial cells in a wound healing assay. **(b)** Actin filament staining (*red*) of migrating epicardial cells

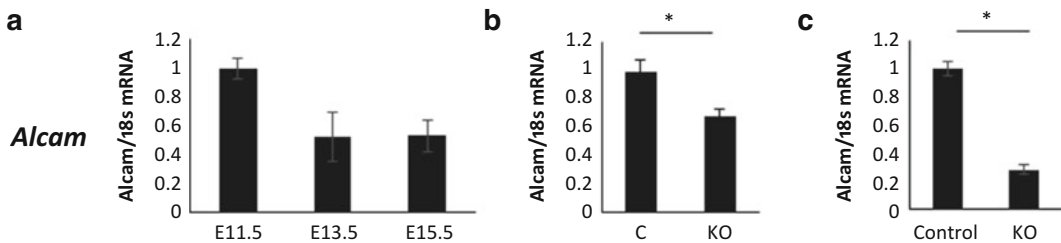


Fig. 3 Gene expression analysis of GFP sorted epicardial cells. **(a)** qRT-PCR analysis of *Alcam* in GFP+ epicardial cells at different days of development. **(b)** qRT-PCR analysis of freshly isolated GFP+ epicardial cells using FACS from *Gata-5 Cre-1/Wt1loxP/gfp* (C) and *Gata-5 Cre+/Wt1loxP/gfp* (KO) mice. **(c)** qRT-PCR analysis of GFP+ immortalized control and *Wt1KO* epicardial cells. Arbitrary Units are used

The embryonic epicardium constitutes a heterogeneous population of cells that dynamically changes during heart development; taking into account this fact an accurate characterization of epicardial cells at each embryonic stage will help us understand their nature and composition. The possibility to generate immortalized epicardial cells at each embryonic stage using the methods described in this chapter could represent an interesting alternative.

We believe that the combination of independent approaches using in vitro immortalized epicardial cells, freshly isolated GFP sorted epicardial cells, and *epiWt1KO* cells is required to demonstrate new functions of *Wt1* in the embryonic epicardium (Fig. 3). The present chapter describes diverse strategies for the generation of immortalized epicardial cells from different mice in order to

study the role of *Wt1* during epicardium development. It also describes the enzymatic digestion of embryonic GFP hearts from *Wt1GFPKI* mice and control and *epiWt1KO* mice.

1.1 Equipment

1. Dissecting fluorescent stereomicroscope.
2. 33 °C and 37 °C incubators containing 5% CO₂.
3. Water bath.
4. Microcentrifuge.
5. Thermomixer (Eppendorf).
6. Surgical materials: forceps and needles.
7. Laminar cell culture hood
8. Epifluorescent or confocal microscope for visualizing cells.
9. Plate reader (485 nm excitation and 520 nm filters).
10. FACS machine (FACSAria™).
11. LC480 real time PCR machine (Roche).

2 Materials

2.1 Cell Culture Reagents

1. Gelatin solution: 100 mg of gelatin dissolved in 100 ml of sterile water. Autoclave and store at 4 °C.
2. Trypsin solution: Trypsin diluted 1:10 in Versene (0.48 g/l EDTA in PBS and 6 ml/l of Phenol Red Solution 0.5% in PBS).
3. Tamoxifen stock solution: dissolve 20 mg tamoxifen in 1 ml of ethanol. Store at -80 °C.
4. Tamoxifen working solution: dilute the tamoxifen stock solution 50 times in DMEM medium without serum. Store at -20 °C.
5. Glutamine solution: l-Glutamine Solution 200 mM (Sigma G7513).
6. Antibiotic solution: Penicillin/Streptomycin.
7. Cell culture condition: Dulbecco's Modified Eagles Medium (DMEM) containing GlutaMAX (Life Technologies), 4.5 g/l d-glucose and pyruvate, and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Store at 4 °C.
8. Mouse interferon gamma: Dissolve 1 vial of mouse interferon gamma in DMEM medium without serum at 20 µg/ml. Keep aliquots frozen at -20 °C.
9. PBS (Phosphate Buffered Saline): 138 mM NaCl, 2.7 mM KCl, 8.2 mM Na₂ HPO₄, 1.5 mM KH₂ PO₄.

2.2 Transfection Reagents

1. Lipofectamine 2000.
2. Optimen medium.
3. CAGGs-CreERT2-IRES-puro R expression construct.

2.3 Immuno-fluorescence Reagents

1. Round glass coverslips coated with 1 % gelatin solution.
2. 2 % Paraformaldehyde solution (PFA): prepare 2 % PFA solution in PBS, pH 7.4. Work in a chemical hood. Keep aliquots frozen at -20°C .
3. Humidified chamber: prepare a humidified light-protected chamber covered with aluminum foil enough to keep a 24-well plate.
4. Permeabilization solution: 0.1 % Triton X-100 in PBS.
5. Blocking solution: 2 % (w/v) bovine serum albumin (BSA) in PBS.
6. Primary antibody at appropriate dilution in blocking solution.

2.4 Migration Assay

1. Calcein AM: To make 2 mM stock solution, dissolve 1 vial (50 μg) in 30 μl of DMSO.
2. Calcein working solution: Dissolve stock solution to make a 2 μM working solution.
3. 24-well transwell membranes with 8 μm pores.

2.5 FACS Reagents

1. Trypsin solution: Trypsin diluted 1:10 in Versene (0.48 g/l EDTA in PBS and 6 ml/l of Phenol Red Solution 0.5 % in PBS).
2. DMEM culture medium containing GlutaMAX (Life Technologies), 4.5 g/l d-glucose, 1 % pyruvate, and supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Store at 4°C .
3. RNAlater buffer solution (Ambion)

2.6 qRT-PCR

1. For Taqman[®] real time PCR assays use: LC480 2 \times real time master mix (Roche), RNase/DNase free water, Taqman[®] probes and endogenous *Gapdh* mouse housekeeping control assay (Roche). Probes and primers are selected according to the universal probe library primer/probe design tool (Roche).
2. For SYBR green real-time assays use: GoTaq 2 \times real time master mix (Promega), RNase/DNase free water, primers that produce products less than 200 bp (design using Primer3 software), and 18s primers as a housekeeping control (Biomers).

3 Methods

3.1 Mouse Breeding

To generate immortalized epicardial cells *Wt1+/*gfp** mice were crossed with the H-2KbtsA58 “immorto” mice carrying a temperature-sensitive simian virus 40 (SV40) large-T antigen 20.

Wt1gfp/+/Immorto^{+/-} were mated with *Wt1+/-*, *Wt1loxP/loxP* or CAGG-CreER; *Wt1loxP/loxP* mice in order to generate *Wt1gfp/+* (Control) and *Wt1gfp/-* (*Wt1KO*), *Wt1gfp/loxP* (*Wt1loxP* cell lines) or tamoxifen inducible KO (Cre⁺) (*CreER+/Wt1loxP/gfp*) and control cell lines (Cre⁻) (*CreER-/Wt1loxP/gfp*) [3, 7, 8]

3.2 Generation of Mixed Population of Immortalized Epicardial Cells

1. Coat 24-well plates by pipetting 0.5 ml of 1 % gelatin solution, allow to stand for at least 30 min and aspirate.
2. Dissect out embryos into sterile PBS and select WT1-GFP positive embryos under a fluorescent stereomicroscope. Fluorescence can be clearly seen in the embryonic kidney as a positive control.
3. Dissect out hearts (also tails for genotyping if needed) and cut out atria with sterile needles.
4. Place GFP+ heart ventricles in a 24-well gelatin dish and incubate. Epicardial cells can be seen migrating out of heart explants onto the surface over the gelatin within 24 h after the procedure.
5. After 48 h, carefully transfer the 24-well plate to the culture hood and remove ventricles with a needle and allow cells to reach confluence and propagate at 33 °C.
6. Cells are propagated at 33 °C in DMEM containing 10 % FCS and 20 ng/ml mouse gamma interferon (Peprotech).

3.3 Generation of Cre⁺; Wt1^{loxP/gfp} Stables Clones

1. Day 1: Trypsinize subconfluent *Wt1^{loxP/gfp}* cells and seed at a density of 40,000 cells per cm² in a 24-well plate overnight.
2. Day 2: Transfect each well with 1 µg of CAGGs-CreERT2-IRES-puro R expression construct following lipofectamine 2000 instructions.
3. Day 3: Expand cells by transferring cells onto P100 cell culture dishes.
4. Day 4: Add selection antibiotic (5 µg/ml puromycin).
5. Feed cells with fresh medium containing antibiotics every second day until isolated colonies appear.
6. After 2 weeks, pick healthy colonies with Whatman paper discs and expand them for further analyses.

3.4 Characterization of Immortalized Cells by Immunostaining

1. Trypsinize subconfluent immortalized cells and seed at a density of 40,000 per cm² in a 24-well plate to which sterile coverslips have been coated with gelatine for at least 30 min.
2. Allow cells to grow until desired confluence is reached over the coverslips.
3. Remove medium and fix cells with 2 % PFA for 10 min, followed by three washes with PBS.

4. Permeabilize cells for 3 min with PBS containing 0.1% Triton X-100.
5. Block cells with PBS containing 2% BSA for at least 1 h at room temperature.
6. Incubate overnight with primary antibody at an optimal dilution.
7. Aspirate primary antibody and wash with PBS three times for 5 min each.
8. Incubate for 1 h with secondary antibody at room temperature in the dark.
9. Wash three times with PBS.
10. Add Vectashield containing DAPI and mount coverslips. Use nail polish to seal the edges of the coverslips.
11. Acquire images using epifluorescence or confocal microscopy.

3.5 Functional Assays

3.5.1 Wound Healing Assay

1. Trypsinize subconfluent immortalized cells and seed at a density of 80,000 cells per cm² on a gelatine coated 24-well plate. Leave overnight at 37 °C.
2. Using a P200 pipette tip incise a wound in the central area of the confluent 24-well culture.
3. Gently wash the cells twice with DMEM medium without FCS to remove detached cells.
4. Add fresh warm (37 °C) DMEM medium containing 1% FCS and the desired additives and conditions.
5. Incubate cells at 37 °C for 24 h and capture images using a live cell imaging system.

3.5.2 Transwell Migration Assay of Immortalized Epicardial Cells

1. Trypsinize subconfluent immortalized cells and seed at a density of 40,000 cells per cm² in a T25 flask the day before the experiment. Incubate overnight.
2. Starve cells in culture by adding serum free medium overnight.
3. Coat 24-well transwell membrane inserts with 8 µm pores by adding 200 µl in the apical zone and 900 µl in the basal zone with 1% gelatin solution. Incubate at 37 °C for 1 h.
4. Wash both sides of the insert with PBS three times.
5. Block membrane by adding 5% BSA in PBS overnight at 4 °C.
6. Next day wash inserts with sterile water and leave to dry for 3 h inside a hood.
7. Trypsinize starved cells, centrifuge, and resuspend in DMEM medium with 1% BSA.
8. Seed cells on gelatine coated 24-well transwell membrane inserts at a density of 8 × 10⁵ cells per well. Seeding volume 0.3 ml per insert (upper chamber).

9. Allow cells to migrate towards your “selected conditions” present in the lower chamber (0.9 ml) for 8 h at 37 °C, include a positive control medium containing 10% FCS and negative control with medium without serum.
10. Gently, aspirate medium from both lower and upper chambers and wash twice with PBS.
11. Add Calcein containing solution to each well, 0.1 ml to the upper part and 0.4 ml to the lower part.
12. Incubate cells at 37 °C in a CO₂ incubator for 30 min and wash with PBS.
13. Transfer to a new 24-well plate containing 0.9 ml of DMEM medium with 1% BSA.
14. Read fluorescence in a plate reader (485 nm excitation and 520 nm filters).

3.6 Generation and Analysis of GFP Sorted Epicardial Cells from *Wt1GFPKI* Mice and Control and *epiWt1KO* Hearts

1. Dissect out embryos into fresh PBS and select *Wt1GFP* positive embryos using a fluorescent microscope. Fluorescence is clearly seen in the embryonic kidney as a positive control.
2. Dissect hearts (also tails for genotyping if needed) and cut out atria with sterile needles. Take a GFP negative heart as a negative control for fluorescence.
3. Wash dissected ventricles three times with PBS.
4. Warm up trypsin solution: trypsin:versene solution in a 1:10 ratio warmed up to 37 °C.
5. Place GFP heart(s) in a 1.5 ml Eppendorf tube and remove PBS.
6. Add 200 µl of trypsin solution.
7. Place Eppendorfs in a Thermomixer set at 37 °C and mix at 1000 rpm for 10 min.
8. Collect supernatant with dissociated cells into a separate tube containing DMEM medium (with added 10% FCS and 1% Penicillin/Streptomycin) and add fresh trypsin solution. Repeat **steps 7 and 8** until heart is completely dissociated. Keep supernatant on ice until all dissociated cells are collected.
9. Pass collected cells through a sterile sieving membrane or cell strainer before FACS analysis and sorting.
10. FACS sorting of GFP populations by gating against a littermate GFP negative control.
11. Collect cells directly into RNAlater buffer solution (Ambion) or Trizol, centrifuge gently and store pellet at -80 °C.

3.7 qRT-PCR Quantification

1. Extract RNA from collected cells and measure RNA concentration and quality. We use an RNA extraction kit (Qiagen or Arcturus for very small samples). Store RNA at -80 °C until enough RNA is available to proceed.

2. *Optional. In order to reduce the amount of RNA/samples needed you can amplify the RNA. For RNA quantities of around 5 ng the Arcturus RiboAmp HS-Plus two-round and Turbo labeling kit has been used in our laboratory following the manufacturer's instructions.
3. Convert RNA or amplified RNA into cDNA using Superscript III reverse transcriptase (Invitrogen) following manufacturer's instructions.
4. Sybr Green (Gotaq, Promega) and Taqman (Roche) reaction master mixes for quantification could be used.
5. Select a housekeeping control assay such as 18s or the endogenous mouse *Gapdh* (Roche) for Taqman quantification.
6. Carry out a serial dilution of a control template and dilute samples for quantification.
7. Run qRT-PCR and generate C_t values using an optimized thermal profile.
8. Plot standard curve and read off values from quantified samples.

4 Notes and Useful Tips

1. When making a wound in a wound healing assay use a rapid movement with the tip to ensure a straight wound.
2. When extracting RNA use filter tips over a working surface free of RNase/DNases. RNaseZAP (Sigma) can be used to clean up bench tops.
3. Trypsin solution works well with embryonic mouse hearts up to E16.5; however E13.5 to E16.5 hearts may need gentle pipetting up and down to enable complete dissociation of cells at the final stages of the procedure.
4. When preparing a PCR reaction avoid cross contamination of samples and ensure thorough mixing of reagents to improve quality of results.

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