Real-Time PCR with DNA from Saliva

Student Laboratory Procedures

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Before you start

Introduction

The purpose of this laboratory exercise is to provide students with a hands-on introduction to the principles and practice of real-time PCR (Polymerase Chain Reaction).

Overview

In this step-by-step laboratory exercise, students will perform the following actions:

A. Collect saliva using non-invasive buccal swabs (1 min)
B. Extract DNA from saliva using a simple heating method (5 min)
C. Mix together DNA and real-time PCR reagents (15 min)
D. Perform real-time PCR using Spartan DX™ instrument (55 min)
E. Analyze DNA results using Spartan DX graphing software (5 min)

Technical Background

DNA from saliva

Human saliva is a rich source of DNA. By microscopy, the most frequently identified cell types in saliva are buccal epithelial cells and white blood cells.

Polymerase Chain Reaction

PCR is an enzymatic method for exponentially amplifying a fragment of DNA (http://en.wikipedia.org/wiki/PCR). The PCR reaction is analagous to a photocopier making millions of copies of a specific page in a book. In this exercise, a fragment of the *human dopamine-D2 receptor* (hDRD2) gene is amplified. The expected fragment size is 124 base pairs.

Real-time PCR

Real-time PCR is a method of simultaneously amplifying and detecting the presence of a target DNA fragment (http://en.wikipedia.org/wiki/Real-time_pcr). In this exercise, the amplified DNA fragments are detected using a DNA-binding dye named SYBR® Green.

Safety

- According to the Centers for Disease Control and Prevention (CDC), universal barrier precautions do not apply to saliva and sputum, unless visibly contaminated with blood
- When handling reagents or preparing PCR mixtures, plastic gloves should be worn to prevent DNA contamination (e.g. Light Labs, Top Glove Nitrile Gloves, Cat. No. Q-2012-L)

Equipment

- Spartan DX instrument
- A heat block set for 65°C
- A heat block set for 98-100°C or a boiling water bath
- Mini centrifuge (e.g. Labnet, Spectrafuge, Cat. No. C1301)
- Pipetters (e.g. Gilson, Pipetman® P, Model P20 and P200)

Materials

- BuccalAmp™ DNA Extraction Kit (EPICENTRE, Cat. No. BQ0901S)
- Kit includes:
  - 15 individually-packaged Catch-All™ Swabs (Cat. No. QEC0925)
  - 15 tubes (1 extraction per tube) of QuickExtract™ DNA Extraction Solution 1.0 (Cat. No. QE09050)
- Mineral oil (PCR-grade) (Biotools, Cat. No. 20.032)
- TAQurate™ GREEN Real-Time PCR Master Mix (EPICENTRE, Cat. No. TM049096)
- 0.2 ml flat-cap PCR tubes (Fisher Scientific, Cat. No. 08-408-214)
- Pipette tips (Light Labs, 200 µl Pipette Tips, Cat. No. B-4053)
- Microcentrifuge tubes (Light Labs, 0.65 ml SureLock tubes, Cat. No. A-7002)
- Sterile water

Order PCR primers (A week in advance)

1. We recommend ordering primers from Integrated DNA Technologies (IDT)
2. Register for a new account (www.idtdna.com)
3. Login to your account
4. Select "Quick Select Standard DNA Oligos"
5. Select "50 nm" as the "Scale" of primer to be synthesized
6. Type in unique primer names
7. Type in the following primer sequences:
    a. Forward primer: 5'-tga act tgg cca cgt tac atg-3'
    b. Reverse primer: 5'-cta ctg tgg gca ttg cac ttt at-3'
8. For purification method, select "Standard Desalting"
9. Click on "Add to Order"
10. Verify order in "Shopping Cart"
11. Click on "Finish Order"

Prepare 100 µM master stock of PCR primers

1. PCR primers will arrive from IDT in dry powder form in a tube
2. On side of tube, you will find the molar quantity of primer e.g. 50 nmol
3. Take the nanomolar value, multiply by 10, and add that volume of sterile water in microliters to the tube e.g. 50 nmol X 10 = 500 µl water to add (primer concentration is now 100 µM)
4. Flick tube for 60 s
5. Centrifuge tube to bring contents back down to bottom of tube
6. Keep tube at room temperature for 24 hrs to fully dissolve primers
7. Aliquot dissolved primers in volumes of 50 µl each in separate microcentrifuge tubes
8. Store primer aliquots in freezer at -20°C for long-term storage

Prepare 10 µM working mix of PCR primers

1. Start with 100 µM master aliquots of forward and reverse PCR primers
2. Take a new microcentrifuge tube
3. Add 5 µl of forward primer and 5 µl of reverse primer to tube
4. Add 40 µl of sterile water
5. Flick tube for 10 s
6. Centrifuge tube to bring contents back down to bottom of tube
7. Each primer is now at a concentration of 10 µM and ready to be used for this exercise

Starting the experiment

Before you start

1. Set one heat block to 65°C and another to 98-100°C or use a slow boiling water bath
2. Turn on Spartan DX instrument and let it pre-heat for 10 minutes

(A) Saliva collection (1min)

1. Label appropriate number of tubes containing QuickExtract solution
2. Rinse mouth twice with water
3. Collect buccal cells by rolling Catch-All swab firmly on inside of cheek, 20 times on each side
4. Place swab end into a tube containing QuickExtract solution
5. Rotate swab end 5 times in the solution
6. Press swab end firmly against side of tube to squeeze out liquid back into tube
7. Screw cap tightly on tube
8. Vortex resultant solution for 10 seconds
9. Proceed to DNA extraction

(B) DNA extraction (5 min)

1. Incubate sample tube at 65°C water bath for 1 minute
2. Flick vigorously for 15 seconds
3. Transfer tube to 100°C and incubate for 2 minutes
4. For long-term storage keep the extracted DNA at -20°C

(C) Real-time PCR (15 min)

1. Flick TAQurate GREEN Master Mix for 5 seconds
2. Centrifuge TAQurate GREEN Master Mix to bring contents back down to bottom of tube
3. Add all of the reagents in Table 1 to a fresh PCR tube
4. Cap tube and flick for 5 seconds
5. Centrifuge tube to bring the contents back down to bottom of tube
6. Open tube and add 15 µl of mineral oil on top of reagent mixture
7. Cap tube and place in Spartan DX instrument

Component	Per 1 reaction (µl)	Per 4.5 reactions (µl)
TAQurate GREEN Master Mix (2x)	10	45
PCR primer mix (10 µM)	1	4.5
Extracted DNA Sample	2	9
Sterile water	7	31.5
Total reaction volume	**20**	**90**

Table 1. Real-time PCR mixture.

(D) Spartan DX (55 min)

1. Set up a cycling program according to Table 2

Step	Temperature	Time	Cycles
Initial denaturation	94°C	135 s	1
Denaturation	94°C	45 s	35
Annealing/extension	53°C	45 s	35

Table 2. Cycling parameters.

2. The instrument screen should look like Figure 1

Figure 1. Screen apperance after the cycling paramenters have been input in the instrument.

(E) Data analysis (5 min)

1. Insert Flash Card into the slot at the bottom of the instrument
2. When program is finished, select "YES" when prompted to "Save Results" to Flash Card
3. Eject Flash Card and insert into a computer that has Microsoft Excel® installed
4. Open Spartan DX graphing program (this program is provided with each instrument)
5. In graphing program, click on "Load Flash Card Data"
6. Find and select your Flash Card data file
7. Graphing program automatically uploads, analyzes, and graphs your data (different graph views are shown on different worksheets in graphing program)

Expected result

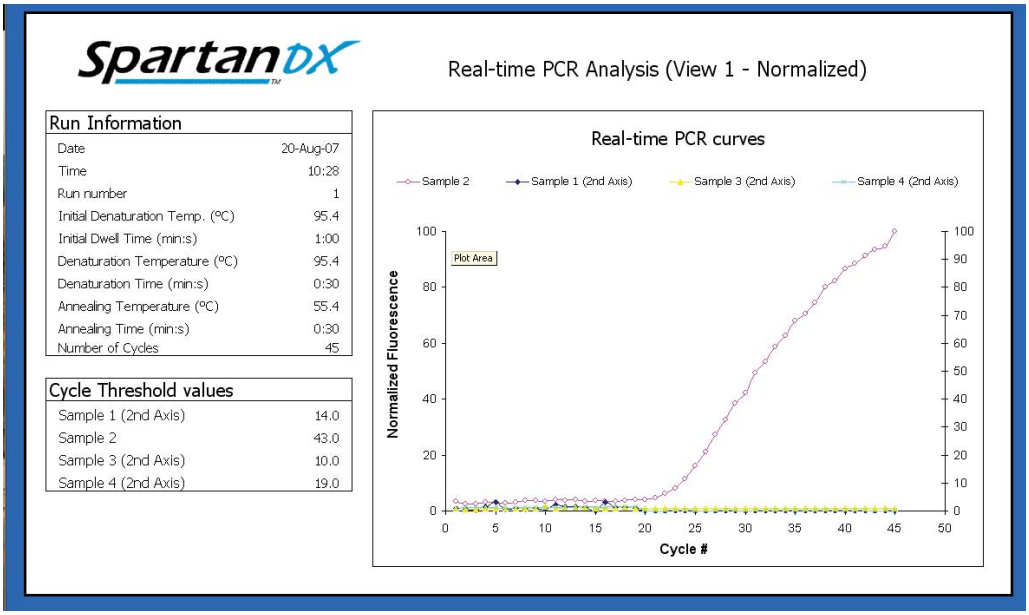

Figure 2. Real-time PCR result.