qPCR with stochastic profiling samples

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Lecture Objectives

qPCR detection method in Janes lab

Primer design and validation

Troubleshooting primers and amplifications
Workflow for qPCR with stochastic profiling samples

Small-sample cDNA amplification

- Extraction of RNA
- Abbreviated RT
- cDNA amplification

Primer Design and Validation

qPCR Setup

Data Output and Analysis

- cDNA reamplification and labeling
- Microarray hybridization and data analysis
What is qPCR?

PCR amplification: Product (P) = Template (T) \times (2)^n
Detection method in Janes lab

SYBR green

Fluorescence increases with concentration of dsDNA. Detects double stranded products, including primer dimers.
Important controls

blank sample, no template control (NTC):
  to check primer dimers and contaminants

no RT sample:
  to check genomic DNA
Whether qPCR reactions have produced single, specific products?

Melting Curves Analysis
Whether qPCR reactions have produced single, specific products?

Agarose gel analysis

- PCR products
- Primer dimers
Primer design

Gene of interest: mRNA RefSeq identification

Homo sapiens jun D proto-oncogene (JUND), transcript variant 1, mRNA
NCBI Reference Sequence: NM_005354.5

Homo sapiens jun D proto-oncogene (JUND), transcript variant 1, mRNA
NCBI Reference Sequence: NM_005354.5
Primer design

Primer3

**Amplicon position:**
within ~400 bp from the 3' end of the transcripts and do not typically span introns, because of the abbreviated RT.

**Amplicon Length:** 150-200bp

Primer length: opt. 20nt

$T_m$: opt 60°C
Primer design

BLAST: Specificity of primers
Primer design

Order 2 sets of primer pairs from a low-cost provider of custom synthesis.

We order 25 nmole with standard desalting. The cost is $0.35 per base in the scale of 15-60 bps.
Primer validation

Verify that the single product is produced: first-stand cDNA sample vs the blank sample in melting curve or gel electrophoresis.

Quantitative accuracy and amplification efficiency:

10-fold dilutions should be 3.3 cycles apart

Slope -3.3 corresponding to amplification efficiencies of 100%
Considerations for qPCR of stochastic samples

Large dilution in H2O to reduce competition from AL1 primer
Troubleshooting primers

Primer dimers:
Detections: melting curve, gel electrophoresis
Troubleshooting primers

Primer dimers:
Detections: melting curve, gel electrophoresis

Solutions:
proper primer design that prevents the formation of hairpins, self dimers, and cross dimers.

decreasing primer concentration two-fold in the RT-qPCR reaction
Troubleshooting primers

Amplification efficiencies of qPCR primer:

Slope of standard curve in the range of -2.9 to -3.9, corresponding to amplification efficiencies (E) of 80-120%
Troubleshooting primers
Amplification efficiencies of qPCR primer:

Low efficiencies:
Main reasons:
bad primer design (2nd structure, not appropriate Tm);
not-optimal reagent concentration.

Solutions:
increasing the primer concentration two-fold in the RT-qPCR reaction
Troubleshooting primers
Amplification efficiencies of qPCR primer: exceedingly high efficiencies:
Reasons:
1. the presence of inhibitors of polymerase enzyme in cDNA samples. Most concentrated samples should be omitted.
2. primer dimers: Decreasing the primer concentration two-fold in the RT-qPCR reaction
3. contamination, inappropriate dilution series
Troubleshooting primers

Pipetting issues:

[Graphs showing Amplification and Standard Curve with data points and regression details]
Troubleshooting primers

Pipetting issues:

use barrier tips

In serial dilution for a standard curve:
1. change tips to prevent the carryover on the tips.
2. not to vortex the tubes so vigorously that the liquid hits the cap and produces contamination when opening it.
Troubleshooting amplifications

qPCR cycle thresholds are all very low ( < 15)
Possible reason: cDNA is overamplified
Solution: Reduce AL1 primer amount or PCR cycle numbers

qPCR cycle thresholds are all very high ( > 25)
Possible reason: RNA in tissue is degraded or amplification is defective
Solution: Perform an amplification with ~100 pg of purified RNA

Possible reason: cDNA is underamplified
Solution: Increase AL1 primer amount or PCR cycle numbers
Anticipated results

Optimized small-sample cDNA amplifications in three distinct biological contexts
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initials, gene names

Standard curve with purified amplicon
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**Optimization with Stochastic Profiling Samples**
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Optimization with Stochastic Profiling Samples
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Human:

housekeeping genes: **GAPDH**, **HINT1**
low abundance genes: **AREG-1**, **ANGPTL4**

Mouse:

housekeeping genes: **mPPIA**, **mGUSB-v2**, **mGAPDH**
low abundance genes: **mEGFR**, **mErbb3**, **mDdr1**

Standard curve with purified amplicon
qPCR setup

4.5 μl of each samples

Standard Curve: dilute 1 μl of amplicon of 449 μl of H2O

Stochastic Profiling Samples: Step 50 in Nature Protocol paper
dilute 1 μl of amplicon of 449 μl of H2O
qPCR setup

3 μl of primer master mix

Fwd primer
Rev primer
H2O
qPCR setup

7.5 µl of 2X RT-qPCR master mix

2 mm Taq DNA polymerase
100x SYBR