Should choose multiple targets

all primers are not exon spanning and have ~60C Tm

all primers were validated by FlyPrimerBank

plan to use qiagen DNeasy blood and tissue kit

"DNeasy Blood & Tissue Kits are designed for rapid purification of total DNA (e.g., genomic, mitochondrial, and pathogen) from a variety of sample sources including fresh or frozen animal tissues and cells, blood, or bacteria."

mtDNA Primers

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Search Term** | **Primer Pair** | **Forward Primer** | **Fwd Pri Temp** | **Reverse Primer** | **Rev Pri Temp** | **PCR Product** | **FBgn** | **Symbol** |
| CoI | PP3681 | CTTGAGCTGGAATAGTTGGAACA | 60.2°C | CAGCGGATAAAGGTGGATAAACA | 60.4°C | 333 | [FBgn0013674](http://flybase.org/reports/FBgn0013674.html) | [mt:CoI](http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl?gname=mt:CoI) |
| ATPase6 | PP11577 | TTTTCTGTATTCGACCCCTTAGC | 60°C | GATCCATTATGACCTGATGGTCC | 60°C | 191 | [FBgn0013672](http://flybase.org/reports/FBgn0013672.html) | [mt:ATPase6](http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl?gname=mt:ATPase6) |
| ND1 | PP5048 | CCTCAACCTTTTTGTGATGCG | 60°C | GACGACCAACCAGCTACTATAAC | 60.2°C | 233 | [FBgn0013679](http://flybase.org/reports/FBgn0013679.html) | [mt:ND1](http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl?gname=mt:ND1) |

nucDNA Primers

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| RPl32 | PD41811 | GCCCAAGGGTATCGACAACA | 60.03°C | GCGCTTGTTCGATCCGTAAC | 59.97°C | 85 | [FBgn0002626](http://flybase.org/reports/FBgn0002626.html) | [RpL32](http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl?gname=RpL32) | Target also used in Salminen 2017 |
| FBgn0003277 | PP13347 | AAGATTTTGCGATGTGTGTGC | 60°C | TGTCCCCTAGACTTCATAACGAT | 60°C | 95 | [FBgn0003277](http://flybase.org/reports/FBgn0003277.html) | [RpII215](http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl?gname=RpII215) | Target also used in Rodríguez 2021 |

Used the Qiagen "Dneasy Blood & Tissue Kit"

Samples were 10 wt larvae frozen at -80

* 1. To Do Homogenize larvae in 180uL of PBS

* 1. To Do Adding 20uL of proteinase K and 200uL of buffer AL
     1. To Do Mix by vortexing or pipetting
     2. To Do Incubate at 56C and vortex periodically until fully digested
     3. To Do Vortex every 10-15mins for 2 hours
        1. To Do I also tried to break up the tissue with a 1000uL pipet periodically
     4. To Do Remove remaining non-soluble fractions by centrifugation at **12,000xg for 10 minutes** and transfer to new labeled microcentrifuge tube

* 1. To Do Add 200uL EtOH, mix by vortexing

* 1. To Do Pipet into Dneasy mini spin column placed in 2mL collection tube
     1. To Do Centrifuge at **6000xg for 1 minute**
     2. To Do Discard flow-through and collection tube
     3. To Do Place spin column in new 2mL collection tube

* 1. To Do Add 500uL AW1
     1. To Do Centrifuge at **6000xg for 1 minute**
     2. To Do Discard flow-through and collection tube
     3. To Do Place spin column in new 2mL collection tube

* 1. To Do Add 500uL AW2
     1. To Do Centrifuge at **20,000xg for 3 minute**
     2. To Do Discard flow-through and collection tube

* 1. To Do Place spin column in a labeled microcentrifuge tube

* 1. To Do Elute DNA by adding 200uL of buffer AE to the spin column
     1. To Do Incubate for 1 minute at room temp
     2. To Do Centrifuge at **6000xg for 1 minute**

* 1. To Do Transfer the flow through to the spin column
     1. To Do Incubate for 1 minute at room temp
     2. To Do Centrifuge at **6000xg for 1 minute**
  2. To Do Nanodrop using buffer AE as blank
  3. To Do Store at -20C for up to 16 years

**Results**

Everything seemed good. Storing in my -20C box

|  |  |  |  |
| --- | --- | --- | --- |
| Sample Name | Nucleic Acid(ng/uL) | A260/A280 | A260/A230 |
| Blank | .3 | 2.08 | -1.27 |
| GRM2 | 89 | 2.14 | 2.25 |
| GRF2 | 71.5 | 2.14 | 2.06 |
| WTM2 | 48.8 | 2.05 | 2.12 |
| WTF2 | 60.3 | 2.1 | 2.2 |
| WTM3 | 28.2 | 2.04 | 1.92 |
| WTF3 | 68.8 | 2.1 | 1.92 |

**Primers**

mtCoI-F 61C

mtCoI-R 62C

RpL32-F 63C

RpL32-R 63C

\*These will be running at suboptimal annealing temps for RpL32

**Sample Dilutions**

**Calculations for 100uL**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Target Conc.** | **Initial Conc.** | **Sample** | **DI** |
| **WTM1** | 5 | 63.9 | 7.82 | 92.18 |
| **WTF1** | 5 | 96.2 | 5.20 | 94.80 |
| **GRM1** | 5 | 45.9 | 10.89 | 89.11 |
| **GRF1** | 5 | 54.7 | 9.14 | 90.86 |
| **WTM2** | 5 | 48.8 | 10.25 | 89.75 |
| **WTF2** | 5 | 60.3 | 8.29 | 91.71 |
| **GRM2** | 5 | 89 | 5.62 | 94.38 |
| **GRF2** | 5 | 71.5 | 6.99 | 93.01 |
| **WTM3** | 5 | 28.2 | 17.73 | 82.27 |
| **WTF3** | 5 | 68.8 | 7.27 | 92.73 |
| **WTM1\_2.5** | 2.5 | 63.9 | 3.91 | 96.09 |
| **WTM1\_10** | 10 | 63.9 | 15.65 | 84.35 |

**qPCR**

**Design the plate layout**

Determine the layout for your plate before pipetting. (can use the template below)

B 
c 
D 
E 
F 
1 
WTMI 
mtCOl 
WTMI 
mtCOl 
WTMI 
mtCOl 
WTMI 
RpL32 
WTMI 
RpL32 
WTMI 
RpL32 
2 
WTFI 
WTFI 
mtCOl 
WTFI 
mtCOl 
WTFI 
RpL32 
WTFI 
RpL32 
WTFI 
RpL32 
3 
GRMI 
mtCOl 
GRMI 
mtCOl 
GRMI 
mtCOl 
GRMI 
RpL32 
GRMI 
RpL32 
GRMI 
RpL32 
4 
GRFI 
mtCOl 
GRFI 
mtCOl 
GRFI 
mtCOl 
GRFI 
RpL32 
GRFI 
RpL32 
GRFI 
RpL32 
5 
WTM2 
mtCOl 
WTM2 
mtCOl 
WTM2 
mtCOl 
WTM2 
RpL32 
WTM2 
RpL32 
WTM2 
RpL32 
6 
WTF2 
mtCOl 
WTF2 
mtCOl 
WTF2 
mtCOl 
WTF2 
RpL32 
WTF2 
RpL32 
WTF2 
RpL32 
7 
GRM2 
mtCOl 
GRM2 
mtCOl 
GRM2 
mtCOl 
GRM2 
RpL32 
GRM2 
RpL32 
GRM2 
RpL32 
8 
GRF2 
mtCOl 
GRF2 
mtCOl 
GRF2 
mtCOl 
GRF2 
RpL32 
GRF2 
RpL32 
GRF2 
RpL32 
9 
WTM3 
mtCOl 
WTM3 
mtCOl 
WTM3 
mtCOl 
WTM3 
RpL32 
WTM3 
RpL32 
WTM3 
RpL32 
10 
WTF3 
mtCOl 
WTF3 
mtCOl 
WTF3 
mtCOl 
WTF3 
RpL32 
WTF3 
RpL32 
WTF3 
RpL32 
11 
WTM1_2.5 
mtCOl 
WTM1_2.5 
mtCOl 
WTM1_2.5 
mtCOl 
WTM1_2.5 
RpL32 
WTM1_2.5 
RpL32 
WTM1_2.5 
RpL32 
12 
WTMI_IO 
mtCOl 
WTMI_IO 
mtCOl 
WTMI_IO 
mtCOl 
WTMI_IO 
RpL32 
WTMI_IO 
RpL32 
WTMI_IO 
RpL32 

**Set up the software**

* 1. First, download and install "Design
  2. and Analysis Software" from thermo at <https://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-3-5-software.html>
  3. Open the software
  4. click "Set Up Plate"
  5. Set Instrument to "QuantStudioTM3"
  6. Set Block to 96-Well 0.2-mL
  7. For a single primer plate, set Analysis to Standard Curve
  8. Select Quantification-SYBR\_with\_Melt

**Run Method**

Set these reaction parameters

Run the reaction:

**Hold**

50oC 2 min

95oC 2 min

**PCR**

40 cycles

95oC 15 sec

59oC 1 min (lowest Tm -5, Thermo Reference suggests 15s)

72oC 1 min

**Melt Curve (default is fine)**

Continuous

95oC 1s

60oC 20s

95oC 1s

**Plate Setup**

Set Passive Reference to none

For each well used in the plate

Enter Sample will be the identifier, ie OreR10ng

Enter Assay will be the primer

Select Reporter is SYBR

Select Quencher is none

Save assay to flash drive

**Set up reagents**

* 1. Dilute the primers to 10μM in water. This should be a 1:10 dilution from the original 100uM stocks.

* 1. Reaction composition (using 10ul per well)

|  |  |
| --- | --- |
| PowerUp SYBR Green Master Mix (2x) | 5μl |
| Forward primer (10μM; 300-800ng total) | 0.5μl |
| Reverse primer (10μM; 300-800ng total) | 0.5μl |
| cDNA template dilution | 4μl |
| Water | 0μl |

* 1. Make a master mix for each primer pair.
     + have 36 wells for each primer (rounding to 38 )

|  |  |
| --- | --- |
| PowerUp SYBR Green Master Mix (2x) | 190μl |
| Forward primer (10μM; 300-800ng total) | 19μl |
| Reverse primer (10μM; 300-800ng total) | 19μl |
| Water | 0μl |

C1 has both primers

C12 and F12 are empty

**Load plate**

* 1. Aliquot 6μl master mix per well
  2. Add 4μl cDNA dilution per well
  3. Mix by pipetting
  4. Remove bubbles by pipetting as best as you can
  5. Cover with adhesive plate cover
  6. Centrifuge plate to remove ALL bubbles
  7. Run plate using assay on flash drive
  8. Save the file to a flash drive, put result file in google Wharton/qPCR folder using this label format yymmdd\_name\_experiment title\_results.eds