# QUANTITATIVE PCR

## Primer Guidelines

1) Optimal primer length is 18-21 bases
2) Optimal product is 150-250 bases
3) Runs of four or more Gs should always be avoided
4) The five nucleotides at the 3' end should not contain more than two G or C bases
5) The Tm of each primer should not differ more than 1-2ºC
6) The Tm of the primer should be 58-60ºC
7) Make a 250nM dilution of primer stock to use for QPCR

## Concentrate Plasmid

1) Use 20-25ml of E. coli culture
2) Prep with Qiafilter midipreps
3) Elute with 70$\mu$l sterile MilliQ
4) Concentrate with PCR kit or ethanol precipitation
5) Resuspend in 30$\mu$l sterile MilliQ

## Standard Curves

1) Calculate the copy number using the following forumula:

   $$\frac{\text{Concentration g/}\mu\text{l}}{\text{Length (bp)} \times 660} \times 6.023 \times 10^{23} = \text{\# molecules/}\mu\text{l}$$

2) Set up standards ($10^{11}$ - $10^{1}$) from stock plasmid

## Reverse transcriptase reaction

1) Start with 1.5$\mu$g RNA in 0.5$\mu$l tubes
2) Make up the following master mix and vortex:

| Volume per reaction ($\mu$l) | Reagent |
|---|---|
| **1.5** | **random hexamers (5$\mu$g/$\mu$l)** |
| **1.5** | **dNTPs (10mM)** |
| **3.0** | **total volume per sample** |

3) Add master mix to each tube.
4) Add $dH_20$ to bring total volume to 18$\mu$l
5) Incubate @ 65ºC for 5min (breaks down 2º structure)
6) Chill on ice for 5min (cold shock to prevent refolding)
7) Make up the following master mix and vortex:

| Volume per reaction ($\mu$l) | Reagent |
|---|---|
| 6.0 | 5x 1st strand buffer |
| 3.0 | 0.1M DTT |
| 1.5 | Rnasin Plus (40u/$\mu$l) |
| **10.5** | **total volume per sample** |

8) Add master mix to each tube (total volume 28.5$\mu$l)
9) Remove 9.5$\mu$l to new 0.5$\mu$l tube
   a. add 0.5$\mu$l $dH_20$ (-RT reaction)
   b. store @ -20ºC
10) Add 1$\mu$l SuperScript II to remaining 19$\mu$l (total volume 20$\mu$l)
   a. Incubate @ RT for 10min
   b. Incubate @ 42ºC for 1hr
   c. Incubate @ 70º for 15min (inactivation)
11) Dilutions for technical triplicates
   a. Remove 8$\mu$l to new 0.5$\mu$l tubes
   b. Add 16$\mu$l $dH_20$ (total volume 24$\mu$l)
   c. Keep on ice

# QPCR reaction

1) Load standards & blanks (-RT reactions, randomly pick a couple)
   a. Make the following master mix:

| Volume per reaction ($\mu$l) | Reagent |
|---|---|
| 10.0 | Dynamo Master Mix |
| 7.0 | $dH_20$ |
| 2.0 | Primer mix (1:100) |
| **19.0** | **total volume per sample** |

   b. Add master mix to each tube (on ice)
   c. Add 1$\mu$l standard (or blank)

2) Load samples
   a. Make the following master mix:

| Volume per reaction ($\mu$l) | Reagent |
|---|---|
| 10.0 | Dynamo Master Mix |
| 5.0 | $dH_20$ |
| 2.0 | Primer mix (1:100) |
| **17.0** | **total volume per sample** |

   b. Add master mix to each tube (on ice)
   c. Add 3$\mu$l sample

**Sample setup**

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | $10^0$ | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ | $10^8$ | $10^9$ | $10^{10}$ | |
| **B** | $wt_0$ | $wt_0$ | $wt_0$ | $mut_0$ | $mut_0$ | $mut_0$ | $wt_{60}$ | $wt_{60}$ | $wt_{60}$ | $mut_{60}$ | $mut_{60}$ | $mut_{60}$ |
| **C** | $wt_{120}$ | $wt_{120}$ | $wt_{120}$ | $mut_{120}$ | $mut_{120}$ | $mut_{120}$ | $B_1$ | $B_2$ | | | | |