Culture preparation

1. Use 10 OD cells: Prepare 25 ml YES cultures with final OD 0.4-0.5
2. Check OD by ODmeter and also count cells in the microscope.
3. Transfer amount of culture necessary for 10 OD to a clean 10-50 ml tube.
4. Centrifuge 2400 rpm 4 mins.
5. Remove supernatant and leave ~1mL. Resuspend cells in the leftover media and transfer to a 1.5 ml tube.
6. Spin down cells and remove the supernatant.
7. Store cells at -80 C. (No wash).

RNA purification (using MasterPure kit)

---- Before you start, set the incubator at 70C and the centrifuge at 4C.
---- Use new gloves and replace them often.
---- Do not talk while working in the RNase-free area.
---- Wipe with RNAseZap: bench area
centrifuge
pipettes
New tip boxes
ice tray and block
New bag of 1.5 ml tubes

----Prepare: isopropanol
70% etOH (with DEPC water)
new bag of 1.5 ml tubes
tip boxes and tips

(Volumes are calculated for 1 sample)

8. Dilute 1 uL of 50 ug/ul ProK (it comes with the kit but its stored at -20C hopper) into 300 ul of Extraction reagent for RNA.

9. Add 300 ul of extraction reagent for RNA containing the Proteinase K to each tube with the cell pellets and mix by vortexing.
10. Incubate at 70 C (in the incubator) for 10-15 minutes; vortex every 5 mins.
11. Place samples on ice for 3-5 minutes and add 175 ul of MPC protein precipitation reagent to 300 ul of lysed sample (solution may become cloudy). Vortex for 10 seconds.
12. Pellet the debris by centrifugation for 10 minutes at 4 C at 10.000 xg.
13. Transfer the supernatant fluid to a clean microcentrifuge tube and discard the pellet.
14. Add 500 ul of isopropanol to the recovered supernatant fluid. Invert 30-40 times.
15. Pellet the RNA by centrifugation at 4C for 10 mins at 10.000 xg.
16. Carefully aspirate the isopropanol without dislodging the RNA pellet.
17. Rinse twice with 70% ethanol, being careful to not dislodge the RNA pellet. Centrifuge briefly if the pellet moves. Remove all the residual ethanol with a pipet.
18. Resuspend the RNA in 35 uL of TE buffer.
19. Measure absorbance by Nanodrop at 230, 260, 280, 260/280 and 230/260 (both ratios should be above 2 for pure RNA).
20. Store samples at -80C.

Determination of genomic DNA concentration by PCR with act1 primers (this quantification method is independent of the Nanodrop quantification)

21. Thaw samples on ice.
22. Make a dilution of the samples: 2 uL sample + 38 uL DEPC water (set **A**).
23. From A, make 1/5 dilution: 4 ul (set A) + 16 ul DEPC water (set **B**); and 1/25 dilution: 2 uL set A + 48 uL DEPC water (set **C**).
24. Set up PCR reaction:

	x1 (uL)
A, B, C	1
Water	13.7
10x PCR buffer	2
dNTPs	1.6
5uM act1 primer mix	1.5
ExTaq	0.2
Total	**20 uL**

25. Run PCR: Program “EXTAQ”

94 C	2:0	
94 C	0:30	x25 cicles
50 C	1:00	
72 C	1:50	
72 C	10:00	
4C	00	

26. Add 5 uL of 5x Orange dye LB.
27. Prepare **1.5 mm** 4% PAGE gels (BioRad Minigel system- 10 ml for each gel):

	x1	x2
30% acrylamide	1.3 ml	2.6 mL
50X TAE	200uL	400 uL
dH2O	8.4 mL	16.8 mL
10% APS	100 uL	200 uL
TEMED	5 uL	10 uL

28. Load gel onto the BioRad Minigel system and use 1X TAE as running buffer.
29. Before running the samples, wash the wells with a syringe three times.
30. Load 2.5 uL of DNA ladder and of each sample with a loading tip.
31. Run for 30 mins at 100 V, or until the samples reach the end of the gel.
32. Prepare a container with 50 mL 1x TAE and 5 uL Sybr Green (1:10000 dilution). The volume must be enough to cover the gel. Ensure the container is protected from light.
33. Carefully remove the gel from the BioRad Minigel system with the help of a spatula and dip it into the Sybr Green solution.
34. Incubate at RT with gentle shaking for 15 minutes and protected from light.
35. Remove the solution carefully without breaking the gels. And wash with diH2O for 15 minutes with gently shaking and covered from light.
36. Repeat the wash 2 more times.
37. Gels now are ready for scanning with STORM system. Make sure to wet the scanner surface before placing the gels and accommodate them carefully to prevent breaking the gels.
38. After scanning, quantify using ImageJ.

Quantification analysis

39. For each mutant strain and each dilution, calculate mutant/wt ratio.
40. Take an average and SD of this ratio of the different dilutions in each strain. (**AVE**).
41. Now, from the Nanodrop quantification data (*step 19*), calculate the volume necessary to obtain 5 ug of DNA for the WT strain (**WT**).
42. Calculate **WT**/**AVE** for each mutant strain to obtain the volume necessary for 5 ug of RNA of each mutant.

DNase treatment

---- Before you start, set the incubator at 65C

	x1
Total RNA (5ug/WT)	___
10x RQ1 DNase I buffer	10 ul
DEPC water	___
RQ1 DNase I	10 ul
RNAse Inhibitor (40U/ul)	0.25 ul
Total	100 ul

43. Incubate at 37 C for 40 mins. (With shaker or shaking every 10 mins)
44. Add 10 ul of Stop reaction buffer and incubate at 65 C for 10 mins.
45. Keep samples on ice from now on. The samples can now be used for qRT-PCR. The rest can be kept at -80C.

qRT-PCR using iScript (Quanta) system

46. Set up qRT-PCR reaction as follows:

Reaction:	x1
Quanta one-step SYBR green Master Mix	12.5
Primer #1 (10 pmol/ul)	1.5
Primer #2 (10 pmol/ul)	1.5
Nuclease-free water	__
RNA template (100 pg)	__

qScript One-step RT 0.5

Protocol

1. 50 C 10 min
2. 95 C 5 min
3. 95 C 10 sec
4. 60 C 30 sec
5. Plate read
6. Go to line 3 for 39 times more
7. 95 C 1 min
8. 55 C 1 min
9. 55 C 10 sec
10. Melting curve from 55 C to 95 C read every 0.5 C, hold 3 sec.