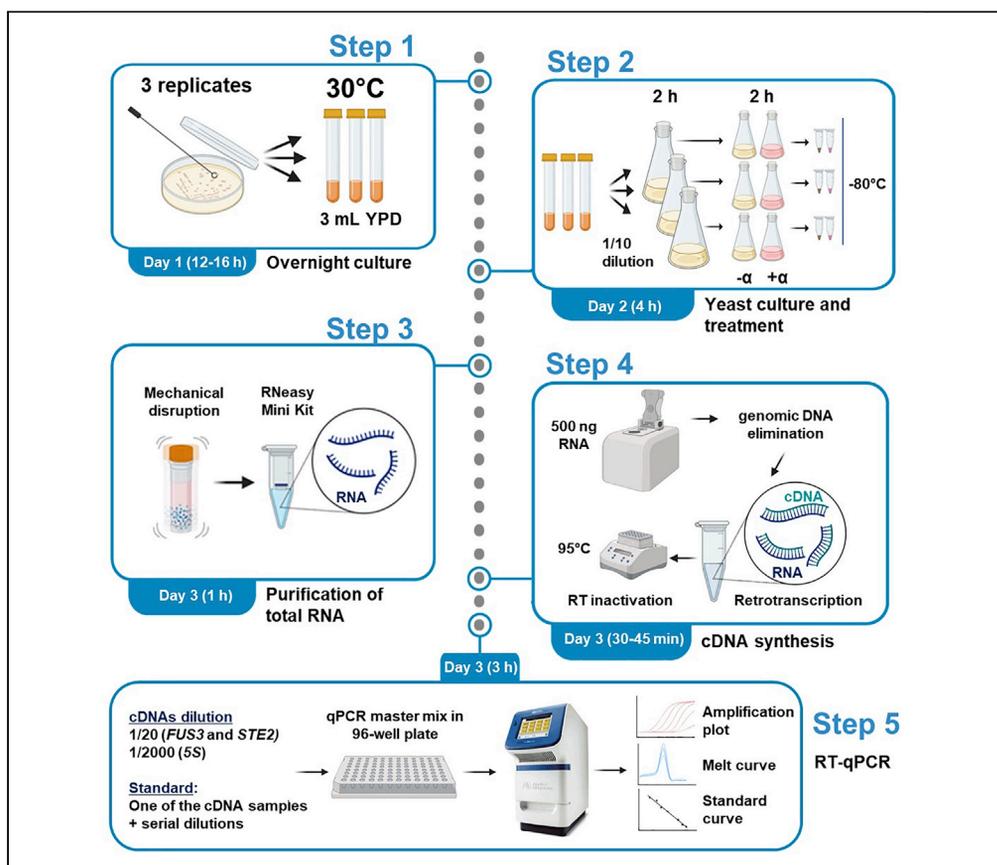


Protocol

Analysis of the pheromone signaling pathway by RT-qPCR in the budding yeast *Saccharomyces cerevisiae*



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Highlights

Optimized procedure to measure the activity of the mating pathway in *S. cerevisiae*

Describes growth conditions in presence or absence of alpha mating factor

Use of RT-qPCR to quantify the expression of mating pathway genes

Can be used to reveal potential regulators of the pheromone signaling pathway

FUS3 and *STE2* expression levels can be used as reporters for signaling through the pheromone pathway in the budding yeast *Saccharomyces cerevisiae*. Here, we describe an optimized protocol to measure the expression levels of *FUS3* and *STE2* using quantitative reverse transcription PCR (RT-qPCR). We describe the steps for comparing untreated and pheromone-treated yeast cells and how to quantify the changes in various deletion strains. The protocol can be applied to determine potential regulators of the pheromone pathway.

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Protocol

Analysis of the pheromone signaling pathway by RT-qPCR in the budding yeast *Saccharomyces cerevisiae*Lucía Ramos-Alonso,¹ Ignacio García,^{2,3} Jorrit M. Enserink,^{1,2,3,*} and Pierre Chymkowitz^{1,4,5,*}¹Section for Biochemistry and Molecular Biology, Faculty of Mathematics and Natural Sciences, University of Oslo, 0316 Oslo, Norway²Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital, 0379 Oslo, Norway³Centre for Cancer Cell Reprogramming, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, 0318 Oslo, Norway⁴Technical contact⁵Lead contact*Correspondence: jorrit.enserink@rr-research.no (J.M.E.), pierrech@ibv.uio.no (P.C.)
<https://doi.org/10.1016/j.xpro.2022.101210>

SUMMARY

***FUS3* and *STE2* expression levels can be used as reporters for signaling through the pheromone pathway in the budding yeast *Saccharomyces cerevisiae*. Here, we describe an optimized protocol to measure the expression levels of *FUS3* and *STE2* using quantitative reverse transcription PCR (RT-qPCR). We describe the steps for comparing untreated and pheromone-treated yeast cells and how to quantify the changes in various deletion strains. The protocol can be applied to determine potential regulators of the pheromone pathway.**

For complete details on the use and execution of this protocol, please refer to Garcia et al. (2021).

BEFORE YOU BEGIN

The budding yeast *Saccharomyces cerevisiae* exists in two mating types known as “a” and “ α ”. Haploid cells can multiply asexually via the mitotic cell cycle, but they can also undergo sexual reproduction, which is the fusion of two cells of opposite mating types (mating) to form a diploid cell, which subsequently can undergo meiosis and sporulation. The ability of cells to mate and sporulate provides a potent survival mechanism during environmental changes in evolving yeast populations (Goddard et al., 2005; Zeyl and Bell, 1997). Mating requires the activation of the yeast pheromone signaling pathway by pheromone molecules, a and α factors, which are secreted by a and α cells, respectively, and bind to receptors expressed on the surface of cells of the opposite mating type (Alvaro and Thorner, 2016). Like all biological systems, there is an inherent level of spontaneous activity in the pheromone signaling pathway, which is often referred to as noise. While low levels of noise are believed to be advantageous for cell populations, allowing small numbers of cells to survive catastrophic events, high noise levels can be detrimental to fitness and have been counterselected in evolution (Kaern et al., 2005; Wang and Zhang, 2011). How cells modulate noise levels in their signaling system is therefore an important research topic in biology. Although noise has been studied in the pheromone signaling pathway (e.g., (Dixit et al., 2014)), it is still not well understood how cells filter noise to accurately respond to the pheromone signals.

Binding of pheromone to its G protein-coupled receptor results in activation of a kinase cascade consisting of Ste20, Ste11, Ste7 and the MAPK Fus3, which subsequently activates a transcription factor program that includes the genes *FUS3* and *STE2* (Oehlen et al., 1996). By monitoring the expression of these mRNAs, we have recently shown that the hypophosphorylated form of the scaffold protein Kel1 is a noise suppressor of the pheromone signaling pathway (Garcia et al., 2021). The



protocol below describes the growth conditions in the presence or absence of pheromone and the analysis of *FUS3* and *STE2* mRNA levels by quantitative reverse transcription PCR (RT-qPCR). This protocol has been performed with MATa-type S288C *S. cerevisiae* cells in presence or absence of α -factor pheromone. We used wild-type, *kel1* Δ and *kel1-ala* (non-phosphorylatable) strains. However, this method can also be used to test different query mutants as potential noise suppressors or regulators of the pheromone signaling pathway, and the protocol can also be applied to monitor the expression of other genes involved in this pathway.

Preparation of materials and solutions

⌚ Timing: 1 h

1. Preparation of a stock solution of α -factor pheromone at 1 mg/mL.
 - a. Dissolve 10 mg of α -factor in 10 mL of ice-cold sterile Milli-Q water (mqH₂O).
 - b. Make aliquots (500 μ L) and store at -20°C .
 - c. Thaw on ice before using and avoid refreezing.
2. Prepare liquid YPD medium and YPD agar plates according to the “materials and equipment” section.
3. Prepare screw-cap-tubes containing 600 μ L of acid-washed glass beads.
 - a. Add 600 μ L of acid-washed glass beads to each 2 mL screw-cap-tube.
 - b. Autoclave at 121°C for 20 min, dry if needed and store at 15°C – 25°C .

Preparation of yeast strains

⌚ Timing: 2–3 days

4. Streak MATa cells of each query strain from a 15% (w/v) glycerol stock stored at -80°C onto an YPD agar plate and incubate at 30°C for 2–3 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
α -Factor Mating Pheromone, yeast	GenScript	Cat# RP01002
Yeast extract	Formedium	Cat# 008013-01-2
D(+)-Glucose anhydrous	Formedium	Cat# 50-99-7
Peptone	Formedium	Cat# 073049-73-7
Agar	Formedium	Cat# 009002-18-0
Power SYBR TM Green PCR Master Mix 2x	Applied Biosystems TM by Thermo Fisher Scientific	Cat# 4367659
2-Mercaptoethanol (β -ME)	Sigma-Aldrich	Cat# M3148
Critical commercial assays		
RNA extraction the RNeasy Mini Kit	QIAGEN	Cat# 74106
QuantiTect Reverse Transcription Kit	QIAGEN	Cat# 205313
Experimental models: Organisms/strains		
<i>S. cerevisiae</i> background S288C. MATa <i>ura3-52, leu2</i> Δ 1, <i>trp1</i> Δ 63, <i>his3</i> Δ 200, <i>lys2</i> Δ Bgl, <i>hom3-10, ade2</i> Δ 1, <i>ade8</i>	(Flores-Rozas and Kolodner, 1998)	WT strain: RDK3023
<i>S. cerevisiae</i> background S288C. MATa <i>kel1::natNT2, ura3-52, leu2</i> Δ 1, <i>trp1</i> Δ 63, <i>his3</i> Δ 200, <i>lys2</i> Δ Bgl, <i>hom3-10, ade2</i> Δ 1, <i>ade8</i>	(Garcia et al., 2021)	<i>kel1</i> Δ strain: JEY11361
<i>S. cerevisiae</i> background S288C. MATa <i>kel1-ala, ura3-52, leu2</i> Δ 1, <i>trp1</i> Δ 63, <i>his3</i> Δ 200, <i>lys2</i> Δ Bgl, <i>hom3-10, ade2</i> Δ 1, <i>ade8</i>	(Garcia et al., 2021)	<i>kel1-ala</i> strain: JEY7758

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
FUS3-F: GAGCTAATGCAGACAGATTTA	Eurofins. (Garcia et al., 2021)	N/A
FUS3-R: CACTTTCACTGCTCTCAAG	Eurofins. (Garcia et al., 2021)	N/A
STE2-F: CCTTCTTGTGGCTTCTATTG	Eurofins. (Garcia et al., 2021)	N/A
STE2-R: CGTCAGCATCAAACCTATC	Eurofins. (Garcia et al., 2021)	N/A
5S-F: GTTGCGGCCATATCTACCAGAAAG	Eurofins. (Garcia et al., 2021)	N/A
5S-R: CGTATGGTCACCCACTACACTACT	Eurofins. (Garcia et al., 2021)	N/A
Software and algorithms		
Applied Biosystems® Real-Time PCR Software	Applied Biosystems™ by Thermo Fisher Scientific	N/A
Other		
Glass beads 0.5 mm dia	BioSpec	Cat# 11079105
Digital Disruptor Genie Vortex	Scientific Industries	Cat# SI-DD38
StepOnePlus™ Real-Time PCR System	Applied Biosystems™ by Thermo Fisher Scientific	Cat# 4376600
2100 Bioanalyzer Instrument	Agilent Technologies	Cat# G2939BA
4200 TapeStation System	Agilent Technologies	Cat# G2991BA

MATERIALS AND EQUIPMENT

Liquid YPD

Reagent	Final concentration	Amount
Yeast extract	1% (w/v)	5 g
D(+)-Glucose anhydrous	2% (w/v)	10 g
Peptone	2% (w/v)	10 g
distilled water (dH ₂ O)	n/a	500 mL
Total	n/a	500 mL

After autoclave sterilization, store at room temperature (RT: 15°C–25°C)

YPD agar

Reagent	Final concentration	Amount
Yeast extract	1% (w/v)	1 g
D(+)-Glucose anhydrous	2% (w/v)	2 g
Peptone	2% (w/v)	2 g
Agar	2% (w/v)	2 g
dH ₂ O	n/a	100 mL
Total	n/a	100 mL

After sterilization, pour into sterile petri dishes and store up to 1 month at 4°C.

STEP-BY-STEP METHOD DETAILS

Yeast cell culture and pheromone treatment

⌚ Timing: Day 1, 12–16 h to grow cells until day 2; Day 2, 4 h until sample collection

The different yeast strains are cultured in three biologically independent replicates and incubated in the presence or absence of α -factor to activate or not the pheromone signaling pathway.

1. Inoculate an individual yeast colony from an YPD agar plate in 3 mL of YPD medium in a 15 mL tube and incubate at 30°C while shaking at 200 rpm for 12–16 h.

2. After 12–16 h, dilute 2 mL of the culture (expect a starting OD₆₀₀ of approximately 10) into 18 mL of fresh YPD medium (1/10 dilution) in a 100 mL Erlenmeyer flask and incubate at 30°C while shaking at 200 rpm for 2 h (expect a final OD₆₀₀ of approximately 2). [Problem 1](#)
3. Split the yeast culture into two 50 mL Erlenmeyer flask (10 mL of yeast cell culture each):
 - a. Add 150 μL of α-factor (1 mg/mL) to one of the yeast cultures (final concentration 15 μg/mL).
 - b. The other culture remains untreated.
4. The incubation continues at 30°C while shaking at 200 rpm for 2 h for both treated and untreated cells.
5. After 2 h of incubation, monitor shmoo formation by differential interference contrast microscopy ([Figure 1](#)). [Problem 2](#)

Note: Shmoo formation is a change of cell morphology that is triggered by the activation of the pheromone signaling pathway.

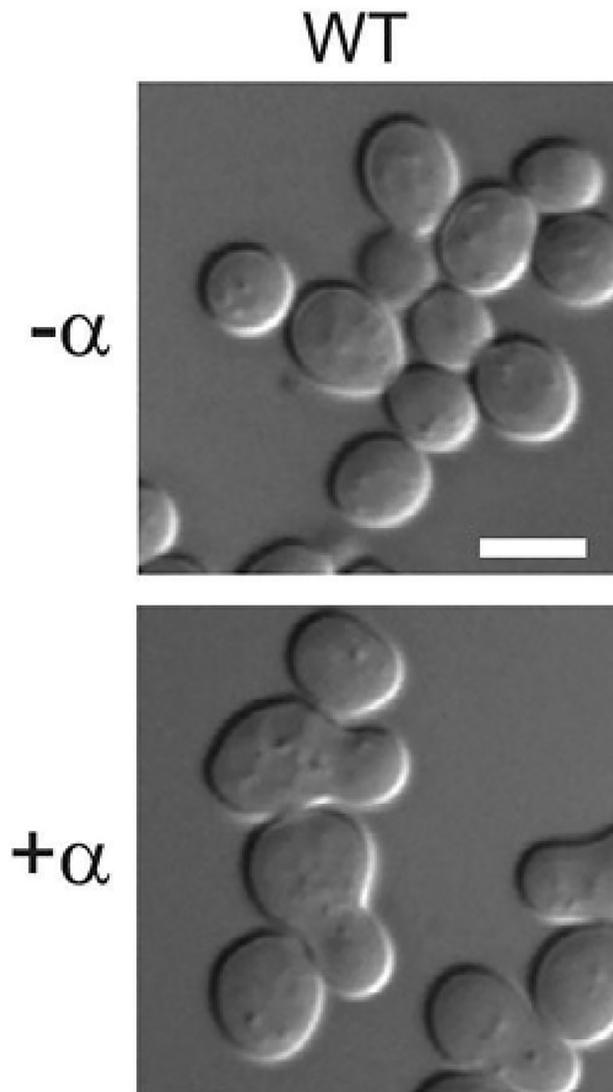


Figure 1. Differential interference contrast images of representative wild-type (WT) cells in absence (top) or presence (bottom) of α-factor

In the lower panel, α-factor has triggered shmoo formation. Scale bar, 5 μM. Adapted from our recently published work ([Garcia et al., 2021](#)) (CC BY 4.0. No changes to the figure were made).

△ **CRITICAL:** Work under sterile conditions. Thaw and maintain α -factor on ice until use. Proceed with step 6 only when shmoo formation has been observed for WT cells.
[Problem 2](#)

6. Harvest yeast cells:
 - a. Centrifuge a volume corresponding to 5 OD₆₀₀ units 5 min at 3000×g.
 - b. Remove supernatant, resuspend the cells using 1 mL of ice-cold sterile mqH₂O and transfer to a new 1.5 mL microcentrifuge tube.
 - c. Centrifuge 1 min at 15,000×g.
 - d. Remove supernatant, snap freeze the microcentrifuge tube in liquid nitrogen and store at -80°C.

△ **CRITICAL:** Step 6c, remove any remaining supernatant before snap freezing.

▣ **Pause point:** Harvested cell pellets can be stored at -80°C for 1 month before proceeding to the purification of total RNA.

Purification of total RNA

⌚ **Timing:** 1 h

RNA purification using adapted steps from the manufacturer's protocol of RNeasy Mini Kit ([RNeasy Mini Handbook - \(EN\) - QIAGEN](#))

△ **CRITICAL:** An appropriate safety protocol has to be followed while handling the RLT and RW1 buffers from the RNeasy Mini Kit: both contain guanidine thiocyanate and β -ME that needs to be added to RLT buffer before use. Wear lab coat, goggles, gloves and use RNase-free filter tips (those will also minimize the risk of RNA degradation). Work under a fume hood to prevent harmful inhalations and use suitable waste containers.

7. Prepare yeast lysate by mechanical disruption:
 - a. Add 600 μ L of RLT buffer (containing 10 μ L of β -M per 1 mL of buffer) to the cell pellets on ice and resuspend by pipetting.
 - b. Transfer the content of the tube to an autoclaved and ice-chilled 2 mL screw-cap-tube already containing 600 μ L of acid-washed glass beads.
 - c. Carefully secure the lid of the tubes and lyse cells at 4°C for 10 min at max speed on a bead mill (Digital Disruptor Genie Vortex, Scientific Industries).

△ **CRITICAL:** β -ME must be added to RLT buffer under a chemical fume hood before use (10 μ L β -ME per 1 mL of RLT buffer). The mixture can be stored at RT for up to 1 month. After the mechanical disruption of the cell walls, the following steps in the purification of total RNA are performed at RT.

Note: QIAGEN recommends not processing more than $\sim 5 \times 10^7$ cells. Then, the above mentioned 5 OD₆₀₀ units harvested as starting material (step 6) may be adjusted depending on the yeast strain background and the spectrophotometer used.

Optional: enzymatic lysis methods using zymolyase or lyticase digestion can be used instead of the mechanical disruption of cell walls ([RNeasy Mini Handbook - \(EN\) - QIAGEN](#)).

8. Transfer the lysate (usually 350 μ L) to a new microcentrifuge tube.
9. Centrifuge 2 min at $\geq 9000 \times g$ and transfer the supernatant to a new microcentrifuge tube.
10. Add 1 volume of 70% ethanol.

11. Mix by pipetting (do not centrifuge) and proceed to step 12.

Note: Precipitates may be visible after addition of ethanol.

12. Transfer the sample (max 700 μ L) including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube.

13. Centrifuge 15 s at $\geq 9000\times g$ and discard the flow-through.

Note: If the sample volume exceeds 700 μ L, centrifuge successive aliquots in the same column.

14. Add 700 μ L of RW1 buffer to the RNeasy spin column.

15. Centrifuge 15 s at $\geq 9000\times g$ to wash the spin column membrane and discard the flow-through.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

16. Add 500 μ L of RPE buffer to the RNeasy spin column.

Note: RPE buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96%–100%) to obtain a working solution.

17. Centrifuge 15 s at $\geq 9000\times g$ to wash the spin column membrane and discard the flow-through.

18. Add 500 μ L of RPE buffer to the RNeasy spin column.

19. Centrifuge 2 min at $\geq 9000\times g$ and discard the flow-through.

Note: The longer centrifugation time in step 19 allows drying the spin column membrane, ensuring that no ethanol is carried over during RNA elution. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through.

20. Discard the collection tube and place the RNeasy spin column in a 1.5 mL RNase-free collection tube.

21. Add 30–50 μ L RNase-free water directly to the spin column membrane, incubate 1 min and centrifuge 1 min at $\geq 9000\times g$ to elute the RNA.

22. Discard the spin column and place immediately the collection tube on ice and proceed with cDNA synthesis. [Problem 3](#)

▮▮ Pause point: Purified RNAs can be stored at -80°C for months. However, it is strongly recommended to immediately proceed to cDNA synthesis to minimize the risk of RNA degradation due to freeze-thaw cycles.

cDNA synthesis (reverse transcription)

⌚ **Timing:** 30–45 min

Reverse transcription was performed using the QuantiTect Reverse Transcription Kit according to manufacturer's instructions ([\(EN\) - QuantiTect Reverse Transcription Handbook - QIAGEN](#)).

⚠ CRITICAL: Set up all reactions and samples on ice to minimize the risk of RNA degradation. Continue using lab coat, gloves and RNase-free filter tips.

23. Quantify RNAs using a NanoDrop spectrophotometer.
24. Prepare the genomic DNA elimination reaction on ice using volumes as listed below.

Genomic DNA elimination reaction components	
Reagent	Amount (per sample)
gDNA Wipeout Buffer 7x	2 μ L
Template RNA, 500 ng	Variable (between 1 μ L and 12 μ L)
RNAse-free water	Variable (up to 12 μ L)
Total	14 μL

△ CRITICAL: Consider dilute the RNAs with RNAse-free water to stay within the range of 500 ng of RNA between a maximum volume of 12 μ L and, to avoid pipetting error, a minimum volume of 1 μ L.

Note: This protocol uses 500 ng of RNA, however, QuantiTect Reverse Transcription Kit protocol works with amounts of template RNA ranging from 10 pg to 1 μ g.

25. Incubate 5 min at 42°C and place immediately on ice.

△ CRITICAL: Do not incubate at 42°C longer than 10 min in step 25.

26. Prepare the reverse-transcription master mix on ice using volumes as listed below and then add the reverse transcription master mix to the template RNA from step 25.

Reverse-transcription reaction components	
Reagent	Amount (per sample)
Reverse transcription master mix	
Quantiscript Reverse Transcriptase	1 μ L
Quantiscript RT Buffer 5x	4 μ L
RT Primer Mix	1 μ L
Template RNA (entire genomic DNA elimination reaction from step 25)	14 μ L
Total	20 μL

Optional: The supplied RT primer Mix will generate random cDNAs but gene-specific primers can be used in a final concentration of 0.7 μ M.

27. Incubate 15 min at 42°C to synthesize the cDNAs.
28. Incubate 3 min at 95°C to inactivate the Quantiscript Reverse Transcriptase and directly perform RT-qPCR.

▮▮ Pause point: cDNAs can be stored at -20°C for months.

RT-qPCRs

⌚ **Timing:** 3 h

Analysis of the expression of *FUS3* and *STE2* by RT-qPCR.

RT-qPCRs were done using a StepOnePlus™ Real-Time PCR System ([StepOnePlus™ Real-Time PCR System \(thermofisher.com\)](#)) and analyzed using the Applied Biosystems® Real-Time PCR Software ([Applied Biosystems® Real-Time PCR Software User Bulletin-for use with StepOne and StepOnePlus \(fishersci.com\)](#)).

Optional: The 5S gene may be used as a control gene because it is not affected by the treatment with α -factor (see [problem 5](#)).

△ CRITICAL: Oligonucleotide primers must be designed so that RT-qPCR products never exceed 200 bp in length.

29. Prepare the following cDNA dilutions for each sample:
 - a. 1/20 dilution (for *FUS3* and *STE2* RT-qPCRs): dilute 5 μ L of cDNA into 95 μ L of DNase-free mqH₂O.
 - b. 1/2,000 dilution (for 5S RT-qPCR): dilute 5 μ L of cDNA 1/20 dilution into 495 μ L of DNase-free mqH₂O.
30. Use one of the undiluted cDNA samples from step 28 to prepare serial dilutions (1/5, 1/10, 1/50, 1/100, 1/500, 1/1000, 1/2000, 1/10000, 1/20000) to generate a standard curve.
31. Prepare the RT-qPCR master mixes on ice. Considering all samples and use a calibration curve for each primer set using volumes as listed below.

qPCR master mix

Reagent	Amount (per sample)
Power SYBR™ Green PCR Master Mix 2 \times	10 μ L
Oligonucleotide-F (Forward, 10 μ M)	0.2 μ L
Oligonucleotide-R (Reverse, 10 μ M)	0.2 μ L
DNase-free mqH ₂ O	7.6 μ L
Total	18 μL

Note: The different cDNA dilutions are adjusted to the expected abundance of each RNA (e.g., for 5S the cDNA dilution used is 1/2,000 as the ribosomal RNA abundance is very high)

32. Pipette 18 μ L per well of the qPCR master mix in 96-well PCR plates suitable for StepOnePlus™ Real-Time PCR System.
33. Add 2 μ L of each cDNA per well (including each dilution of the calibration curve). [Problem 4](#) and [Problem 5](#)
34. Run the RT-qPCR as indicated below.

Standard RT-qPCR cycling conditions for StepOnePlus™ Real-Time PCR System

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	15 s	40 cycles
Annealing / Extension	58°C	1 min	
Melt curve	95°C	15 s	1
	60°C	1 min	
	95°C	15 s	

Note: the melting curve generated with each primer pair indicates the specificity of the PCR reaction. A unique melting peak is desirable and indicates the melting temperature (T_m) of the amplified target.

Table 1. An example of RT-qPCR raw data processing for the gene *STE2*

Experiment	Strain	Gene	Condition	Raw	Mean of WT	Normalized ^a
1	WT	<i>STE2</i>	Untreated	4134.03	4145.927	0.9971
2	WT	<i>STE2</i>	Untreated	4285.48		1.0337
3	WT	<i>STE2</i>	Untreated	4018.27		0.9692
1	<i>kel1Δ</i>	<i>STE2</i>	Untreated	4922.27		1.1873
2	<i>kel1Δ</i>	<i>STE2</i>	Untreated	5350.89		1.2906
3	<i>kel1Δ</i>	<i>STE2</i>	Untreated	4637.47		1.1186
1	WT	<i>STE2</i>	α -factor	9876.19		2.3821
2	WT	<i>STE2</i>	α -factor	7121.5		1.7177
3	WT	<i>STE2</i>	α -factor	10897.7		2.6285
1	<i>kel1Δ</i>	<i>STE2</i>	α -factor	8782.07		2.1182
2	<i>kel1Δ</i>	<i>STE2</i>	α -factor	9652.47		2.3282
3	<i>kel1Δ</i>	<i>STE2</i>	α -factor	9263.19		2.2343

^aNormalized values are calculated by dividing each “raw” value by the “mean of WT” raw values. This sample data was used to generate the plot in [Figure 2](#).

Optional: Alternative and faster cycling conditions are suitable for StepOnePlus™ Real-Time PCR System using other RT-qPCR master mixes.

35. Amplifications are quantified using the standard curve method of the Applied Biosystems® Real-Time PCR Software.
36. Raw data processing and statistical analysis of at least three independent experiments.
 - a. Calculate the mean of WT untreated qPCR results ([Table 1](#)).
 - b. Normalize each qPCR value to the mean of the WT calculated in a ([Table 1](#)).
 - c. Use normalized data to calculate the statistical significance between samples of interest by using the paired-sample Student’s t-test function of the Excel software or the following R-code snippet (<https://www.r-project.org>).

```
# P-value calculation start-----
t.test ( c (Sample1Experiment1,
           Sample1Experiment2,
           Sample1Experiment3),
         c (Sample2Experiment1,
           Sample2Experiment2,
           Sample2Experiment3),
        paired = TRUE
      ) $p.value
# P-value calculation end-----
```

EXPECTED OUTCOMES

The mRNA levels of *FUS3* and *STE2* are known to be upregulated in response to α -factor ([Oehlen et al., 1996](#)). Thus, after the α -factor treatment, a 2 to 4-fold increase of *FUS3* and *STE2* mRNAs levels was observed in the presence of α -factor when compared to untreated cells ([Figure 2](#)). Such an activation of the pathway was observed from WT and mutated cells ([Figure 2](#)) ([Garcia et al., 2021](#)).

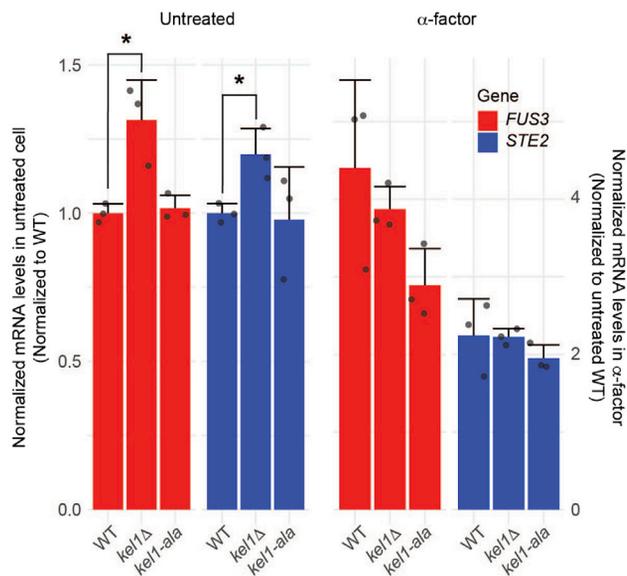


Figure 2. Analysis of *FUS3* and *STE2* mRNA levels in WT and *kel1* mutants in absence and presence of α -factor mRNA levels were normalized to the mean of mRNA levels in WT cells (see step 36 and Table 1). Error bars show the standard deviation of three independent experiments. Statistical significance was calculated using t test, * 0.01 < p < 0.05. Adapted from our recently published work (Garcia et al., 2021).

However, *FUS3* and *STE2* mRNA levels increased in *kel1Δ* cells compared to WT cells in absence of α -factor (Figure 2) (Garcia et al., 2021). This spontaneous activation indicated that Kel1 is a noise suppressor of the mating pathway. In addition, a hypophosphorylated form of Kel1 (*kel1-ala*) repressed the spontaneous pheromone response, as shown by the lack of increase of *FUS3* and *STE2* mRNA levels compared to the WT and *kel1Δ* cells (Figure 2) (Garcia et al., 2021).

In conclusion, this method can be used to test different query mutants as potential regulators of the pheromone signaling pathway.

LIMITATIONS

The specific induction in the mRNA levels of *FUS3* and *STE2* with pheromone compared to untreated cells may vary when analyzing independent experiments. Some pheromone batch-to-batch variations can also be experienced. We always recommend including the WT strain in any future mutant screenings as well to monitor shmoo formation (Figure 1).

TROUBLESHOOTING

Problem 1

Some query yeast mutants may grow slower than wild-type yeasts.

Potential solution

Normally, after 2 h at 30°C under agitation (step 2) the cells should duplicate at least once before starting the pheromone treatment (step 3). To monitor this, measure the OD₆₀₀ before and after the 2 h incubation (step 2). If some of the strains did not duplicate, continue growing them and measure the OD₆₀₀ every 20 min and start the pheromone treatment (step 3) when the OD₆₀₀ has doubled. In the meantime, start the pheromone treatment of the cells that grew at a normal pace (step 3).

Problem 2

Lack of shmoo formation after 2 h of pheromone treatment in WT cells.

Potential solution

Prepare new aliquots of α -factor yeast mating pheromone, increase the final concentration of the pheromone in the yeast medium and/or add a second dose of pheromone after the first hour of treatment. In addition to the WT situation, consider also that some mutants (e.g., *kel1-ala*) form shmoo less efficiently, while others (e.g., *kel1 Δ*) can show aberrant shmoo morphology and/or multiple shmoo even in the absence of pheromone (Garcia et al., 2021).

Problem 3

RNA degradation.

Potential solution

Degraded RNAs are very often responsible for cDNA synthesis and RT-qPCR failures. If RNA degradation is suspected, assess the quality of the RNA before proceeding with the cDNA synthesis. To do so, we recommend to use 2100 Bioanalyzer or 4200 TapeStation systems (Agilent Technologies) to determine the sample integrity and quantity. In any case, a proper handle of the RNeasy Mini Kit, the use of lab coat, goggles, gloves, RNase-free water and filter tips would minimize the risk of RNA degradation.

Problem 4

Low quality or lack of signal in RT-qPCR.

Potential solution

Check the quality of the melting curve and standard curves generated for each primer set. If there is more than one melting peak, if the melting peak is not sharp, or if poor amplification of the standards is observed, it means that the specificity and/or the efficiency of the amplification is low. Consider one or more of the following solutions: prepare new primer dilutions, use less diluted cDNAs, repeat the cDNA synthesis with a higher amount of template RNA, check the integrity of the RNA samples (see [problem 3](#)).

Problem 5

Lack of homogeneity between experimental replicates

Potential solution

Careful harvesting of the same number of viable cells between conditions (step 6) and careful quantification of total RNAs before cDNA synthesis are key measures to reduce sample to sample variation. In addition, a reference gene of which the expression is not affected by different treatment may be used as internal control to normalize RT-qPCR results. Although we usually use 5S ribosomal RNA, other “normalizing genes” may serve as alternatives (Teste et al., 2009).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pierre Chymkowitch (pierrech@ibv.uio.no).

Materials availability

This study did not generate new unique reagents. Yeast strains used in this study appeared in (Garcia et al., 2021) and are available from our laboratory upon request.

Data and code availability

This study did not generate datasets that do not already appear in (Garcia et al., 2021).

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AUTHOR CONTRIBUTIONS

Conceptualization, J.M.E. and P.C.; Writing—Original Draft Preparation, L.R.A.; Writing—Review and Editing, L.R.A., I.G., J.M.E., and P.C.; Visualization, L.R.A., I.G., J.M.E., and P.C.; Funding Acquisition, J.M.E. and P.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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