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A Liquid Assay for Screening Fungal Virulence Factors

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A Liquid Assay for Screening Fungal Virulence Factors

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By

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Abstract

It is difficult to develop drugs against fungal infections due to the fact that both fungi and their hosts are eukaryotic. Using a model host-pathogen system, factors involved in the infection can be identified and studied. By using these two genetic model organisms and this assay in high-throughput screens of genetic knockouts, fungal virulence factors can be identified. We are developing a liquid culture assay for studying the effects of co-culturing *Caenorhabditis elegans* with *Saccharomyces cerevisiae* mutants. We tested several different conditions including different liquid media, *E.coli* to Yeast mass ratios, and *C. elegans* mutant strains. From the results of all the experiments, we concluded that there are other factors that can cause the death of the *C. elegans* in liquid assay which are not *S. cerevisiae* related.

Acknowledgements

We would like to thank Professors Sam Politz and Reeta Prusty Rao for their help, direction and insight on this project. We would also like to thank Charu Jain for her help in the lab. We would also want to thank Meijiang Yun for her help with Sigma Stat and *mek-1* data.

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Introduction

In this project, we are developing a liquid culture assay for studying the effects of co-culturing *Caenorhabditis elegans* with *Saccharomyces cerevisiae* mutants. *Saccharomyces cerevisias* is a unicellular fungus, commonly known as baker's yeast (Daum, 2000). This host-pathogen model system will allow us to do a high-throughput screen of the entire yeast genome. We expect to find the fungal virulence factors involved in fungal infection.

Fungal Infections

Fungi are prevalent in the environment, many of which are pathogens that can infect plants and animals, including humans. For example, fungi are the most common cause of plant disease (Prescott *et al.*, 2005). Fungi such as *Sclerotinia sclerotorium* can cause infection of many crops (Huang *et al.*, 2000). About fifty fungal species have been found to cause disease in humans (Prescott *et al.*, 2005), among which *Candida albicans* is the most common human fungal pathogen (Heitman and Howlett, 2008). Statistics have also shown that more than half of AIDS patients have infections caused by pulmonary *Pneumocystis carinii* (Yang and Kerdel, 2006) and the occurrence of invasive fungal infections in organ transplant patients continues to be high with a high mortality rate (Gabardi *et al.*, 2007). The rise of fungal diseases has many scientists working hard on development of novel antifungal drugs. However, the task has not been easy due to the fact that fungal pathogens are also eukaryotic with cellular machinery very similar to that of their hosts. This makes drug target identification difficult thereby slowing the process of drug development (Heitman, 2005).

Saccharomyces cerevisiae

Saccharomyces cerevisiae, a widely used eukaryotic genetic model, was chosen as the pathogen in this project. *S. cerevisiae* was used instead of a human pathogen, such as *Candida albicans*, because *S. cerevisiae* is the best studied eukaryotic cell (Prescott *et al.*, 2005), with its genome fully sequenced and its biology well understood (Forsburg,

1999). Compared with *S. cerevisiae*, the genomic sequence determination of human fungal pathogen genomes is proceeding very slowly, one reason being that the genome of *S. cerevisiae* is very similar to that of human fungal pathogens, so it is unnecessary to find the genome of the human fungal pathogens (Goffeau *et al.*, 1996). *S. cerevisiae* has 16 chromosomes and a small genome size of about 12 Mbp. About 6000 genes are predicted to be in the genome, and the function of 80% of the predicted genes has been characterized (Miller-Fleming, 2008). Major pathways such as cell cycle, regulation and signal transduction first identified in *S. cerevisiae* are also conserved in higher eukaryotes (Cooper and Hausman, 2007). This has made yeast a very good genetic model for studying higher eukaryotes with more complex genomes. Besides all these advantages, yeast is a safe, fast, and facile organism to culture in the laboratory that makes it an attractive model system. Its fast reproduction cycle accelerates experiment completion (Angier, 1986). Another advantage of using *S. cerevisiae* is that it provides us with an available deletion library of more than 5000 genes that can be used in the screening process of the project.

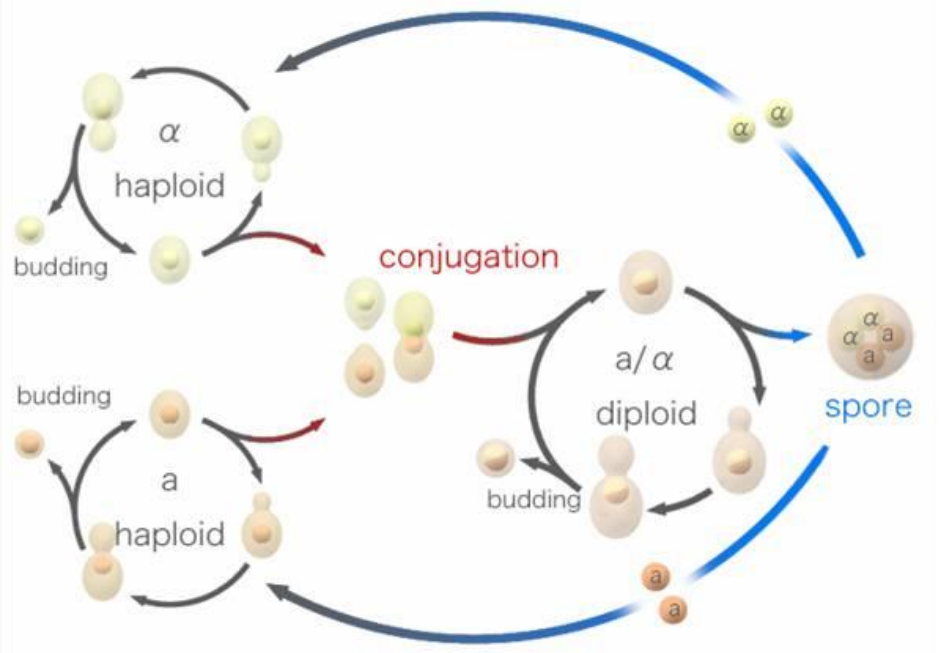


Figure 1 S. cerevisiae Life Cycle

S.cerevisiae has a budding mode of growth. It is normally a diploid with a long G1 phase and overlapped S and M phases. When the cells have enough nutrients, they

will proceed with the cell cycle. When there is a lack of nutrients, the cells will either go into a quiescent state or begin sporulation to become haploid. Haploid cells can go through conjugation to become diploid again (Pringle, 1997).

Caenorhabditis elegans

C. elegan, are free-living in soil and are about 1mm in length. In the lab, they can be maintained on agar plates or liquid cultures. They eat bacteria in the soil and are fed with *E. coli* in the laboratory (Introduction to *C. elegans* Anatomy, 2006).

There are two sexes, hermaphrodite and male. The hermaphrodite is the most abundant sex and males compose only about 0.1% of the time (Introduction to *C. elegans* Anatomy, 2006).

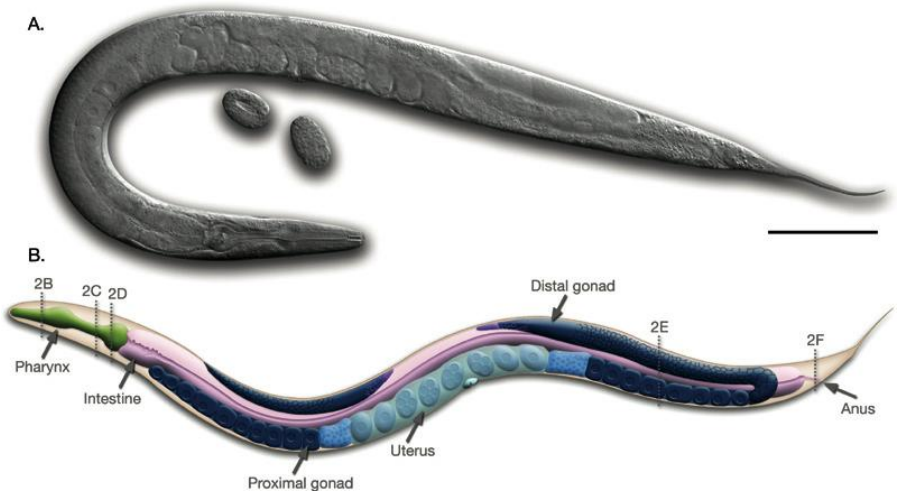


Figure 2: Anatomy of a C. elegans adult hermaphrodite

The main shape is an unsegmented, cylindrical body that is tapered at the ends (shown in Figure 1). In the body wall, there is a cuticle which surrounds the worm. Within the wall there are four different systems; epithelial, nervous, muscle and excretory. The internal organs are incorporated into two systems; alimentary and reproductive. The alimentary includes the pharynx, intestine, rectum and anus. The Reproductive System consists of the somatic gonad, the germ line and the egg-laying apparatus (Introduction to *C. elegans* Anatomy, 2006).

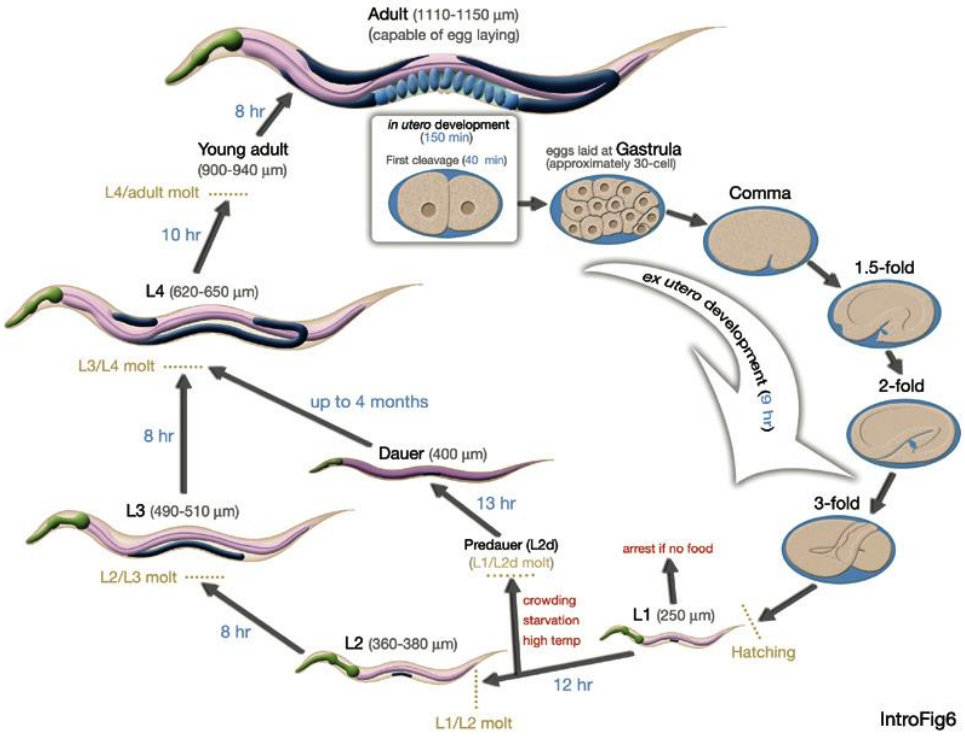


Figure 3: Life cycle of C. elegans

Figure 3 shows the life cycle of a C. *elegans* hermaprhodite. This entire cycle takes only three days at 20 degrees C. The stages of the life cycle are the embryonic stage, larvae (L1-L4) stages, and adult (Introduction to *C. elegans* Anatomy, 2006).

The embryonic stage can be separated into two parts; proliferation and organogenesis/morphogenesis. During the four postembryonic larval stages, the development of the nervous system and the reproductive system occurs. If the egg hatches and there is no food available, the larva stays at the L1 stage. The adult is the fully developed reproductive form. (Introduction to *C. elegans* Anatomy, 2006).

The dauer larva is formed under conditions of food limitation, starvation, or a high temperature. This stage is non-aging if the environment stays unfavorable for growth. When the dauer larva is exposed to food or a more favorable environment, then the worm exits the dauer stage and develops to the L4 and then into an adult, (Introduction to *C. elegans* Anatomy, 2006).

Model Host

C. elegans has been used to provide important insights into how animals perceived threats in the environment and defend themselves against infection. This nematode has been very useful in many different aspects in biology such as developmental biology, neurobiology, and ageing (Gravato-Nobre and Hodgkin, 2005).

C. elegans is an ideal model host for many reasons. From the practical standpoint the advantages are low cost, easy maintenance, minimal lab space, and suitability for automated animal sorting (Gravato-Nobre and Hodgkin, 2005).

They also have a small genome size. This has helped scientists to determine the complete DNA sequence of the genome. Self-fertilization is an important aspect of *C. elegans* for genetic analysis. Another aspect that is important for studying development is that their body is transparent allowing observation of all cells and cell divisions from egg to adult. This also makes it easier to observe the infection process (Gravato-Nobre and Hodgkin, 2005).

Either in the soil or in the laboratory, *C. elegans* eats bacteria, which constitutes a convenient route for infection by microbes, as well as the application of RNA interface-based gene silencing (Gravato-Nobre and Hodgkin, 2005).

C. elegans is a very important model in studying innate immunity. There are at least four different pathways that are involved in the innate immunity; DBL-1, DAF-2/DAF-16, p38 MAP kinase, and ERK. There is a common theme with all the pathways, in that the signaling pathways involved in innate defenses involve protein cassettes that play essential roles in other, seemingly unrelated developmental or physiological processes (Ewbank, 2006).

Previous Projects in the Politz and Prusty Rao laboratories

Previous studies have identified *S. cervisiae* mutants that are virulent as well as worm mutants that show altered susceptibility to yeast infection. It was found that *S. cerevisiae* could infect *C .elegans* and induce the Dar phenotype. *C. elegans* produces reactive oxygen species (ROS) which are produced by the product of the *bli-3* gene, a

NADPH-dependent oxidase required for normal cuticle formation. Preliminary evidence suggests that production of ROS by BLI-3 may protect *C. elegans* against yeast pathogenesis (Charu Jain and R. Prusty Rao, unpublished). *S. cerevisiae* may then use gene products of YAP 1 and SOD 1 to neutralize the effect of ROS. YAP 1 is a transcription factor in *S. cerevisiae* that is critical for oxidative-stress response (Maeta *et al.*, 2004). *SOD1* encodes the protein superoxide dismutase, which can neutralize ROS by converting superoxide radicals to hydrogen peroxide and molecular oxygen (Cox *et al.*, 2002).

These results suggest that a high-throughput assay would allow us to conduct an unbiased whole genome screen for novel fungal virulence factors as well as host immunity modulators. However, experiments on agar plates are labor intensive and time consuming. In order to screen the deletion libraries of *S. cerevisiae*, a more efficient assay is needed. We decided to try to develop a liquid culture assay in microtiter plates.

Methodology

Stock Plate Maintenance

The stocks of *C. elegans* strains were kept on 60 mm agar plates of NGM medium with an *E. coli* OP50 lawn at 16^{o}C. Three hermaphrodites were transferred every 5 to 7 days to fresh plates. The plates were transferred to 20^{o}C four days before egg preparations were done (Kelly and O'Brien, 2007).

Mutant C.elegans

Fer-1 mutant *C. elegans* is temperature-sensitive fertilization defective. At 20 $^{\circ}$C, fer-*1* hermaphrodites are infertile. The *fer-1* gene product is required for Ca^{2+}-mediated membrane fusion during *C.elegans* spermiogenesis. *Fer-1* mutation alters the sensitivity to Ca^{2+}, which affects the membranous organelle fusion and results in abnormal sperm (Washington and Ward, 2006).

C. elegans Egg Preparation

Egg preparations were used to start developmentally synchronous liquid cultures in microtiter wells. Use of egg preparations also helped avoid contamination due to the use of bleach treatment to lyse worms, which sterilized the egg preparation. To make sure the worms were exposed to yeast are at the same stage of development, eggs were harvested as follows.

The egg preparation stock plates were prepared three to five days earlier to obtain the maximum number of eggs on plates. Plates were not used if they did not contain enough eggs or if the plates had starved. These plates were washed with M9 buffer to remove eggs and worms. Then the liquid was transferred to a 15mL conical tube. It was centrifuged into a pellet for two minutes at 900xg. The supernant was removed using a Pasteur pipette. Twelve milliliters of bleach solution, which consisted of 0.25M NaOH dissolved in a 1:4 dilution of commercial bleach in dH_2O, was added to the conical tube to lyse the worms. The eggs were more resistant to the bleach solution, so they stayed intact. The tube was rocked for three minutes and centrifuged for another two minutes at

900xg. The superntant was removed quickly, and then sterile M9 was added to wash the eggs free of traces of bleach. Between washes, samples were centrifuged for two minutes at 900xg and the supernatants were removed.

After the second wash, the pellet was resuspended in 5 mL of M9 buffer. Three 10µL drops were added to an unused agar plate to determine the average egg count for a giving volume. A volume containing about fifteen to twenty eggs was then transferred into the microtiter wells and incubated at 20^{o}C to mature.

Preparation of Liquid Assay

A single colony from a streaked plate of *E. coli* OP50 was added to 5 mL of LB and incubated in 37^{o}C for three days. A single colony from a streaked plate of *S. cerevisiae,* such as RPY101 strain, was added to 5 mL of YPD and incubated at 30^{o}C for three days.

After the three days, the tubes were removed from the incubators and the absorbance at _600__ nm was taken. For *S. cerevisiae,* YPD was used as a blank and for *E. coli,* LB was used as a blank. The dilution for the absorbance was 1:10. It is important to take into account that *S. cerevisiae* yeast cells are very large compared to *E. coli* cells. To determine the number of cells per milliliter for *S. cerevisiae* and *E. coli*, the absorbance that was determined was converted to cells/mL using the factor 8 x 10^8 cells/OD unit for *E. coli* and 2 x 10^7 cells/OD unit for *S. cerevisiae,* also taking into account the dilution factor, which was 10.

The mass ratio was determined by divided the calculated value of *S. cerevisiae*'s cells/mL by the calculated value of *E. coli'*s cells/mL, then multiply the result by 10 since one *S. cerevisiae* cell weigh 10 times more than an *E.coli* cell.

The total volume of liquid in each well was 750 µL. The volume of S. media added equals to the total volume minus the volume of *S. cerevisiae* and *E. coli* together. To each well there was an addition of 1 µL streptomycin 50 mg/mL to stop the growth of *E. coli* and bacterial contaminants. Components were added to each sample in the amounts indicated in Table 1.

Component	Added Amount
E. coli	50 µL
Yeast	15 µL - 150 µL (depending on absorbance and concentration)
S. medium	750 µL – (yeast + *E. coli*)
Streptomycin	1 µL of 50 mg/mL
Eggs	5-10 µL (depending on the egg count)
Total Volume	750 µL+ egg volume + Strep volume

Table 1: Assay Set-Up

Assay Procedure

The microtiter plates were incubated at 20^{o}C for ten to twelve days, during which observations and data were recorded daily. The total number of live and dead worms was recorded. Worms, that were not moving or that had larvae hatched inside of bodies, were considered to be dead.

Data Analysis using Sigma Stat

A computer program called Sigma Stat was used to plot survival percentage as a function of time. The statistics that were used is called LogRank. This compares the survival distributions of two samples. It constructed by comparing the observed and expected number of events in one of the groups at each observed event time and then adding these to obtain an overall summary across all time points where there is an event.

Data were arranged in three columns; group, time and status. The group was each condition to be compared. Time was the number of days since plating of eggs. Status was alive or dead. For each day, each death was recorded in a separate line in the spreadsheet. Each worm had to be entered in as an individual. For every worm that had died that day there would be a one for the status and the number of rows for that day would coincide with the number of worms that died that day. For each row there would be the group name, the day, and a one for the status. If no worms died within a 24 hour period then there would be a group name, the day, and for the status it would be left blank.

To plot the graph and calculate significance between groups, the log-rank test was used. Groups to be compared pairwise were chosen, and because worms could not escape the microtiter wells, no data were censored (the set-up is also shown in Appendix 2).

Results

All the Figures in this section are survival curves plotted using Sigma Stat.

C.elegans arrested at L1 stage when *S. cerevisiae* served as the only food

In wells that *C.elegans* were fed with 100% *S. cerevisiae*, the eggs hatched but were all arrested at L1 stage in development (data not shown). This is consistent with results from previous projects on agar plates.

S. cerevisiae Mutants

Wild type *S. cerevisiae* (*Y101), S. cerevisiae SOD1* mutant and *Sigma* strain *(Y196)* were used in this experiment. *Sod1Δ* is a superoxide dismutase deletion mutant. *Sigma* (*Y196)* is a wild strain that is more virulent than *Y101*. The mass ratio of all *S. cerevisiae* to *E.coli* was 1:30. NGM was used in all the wells.

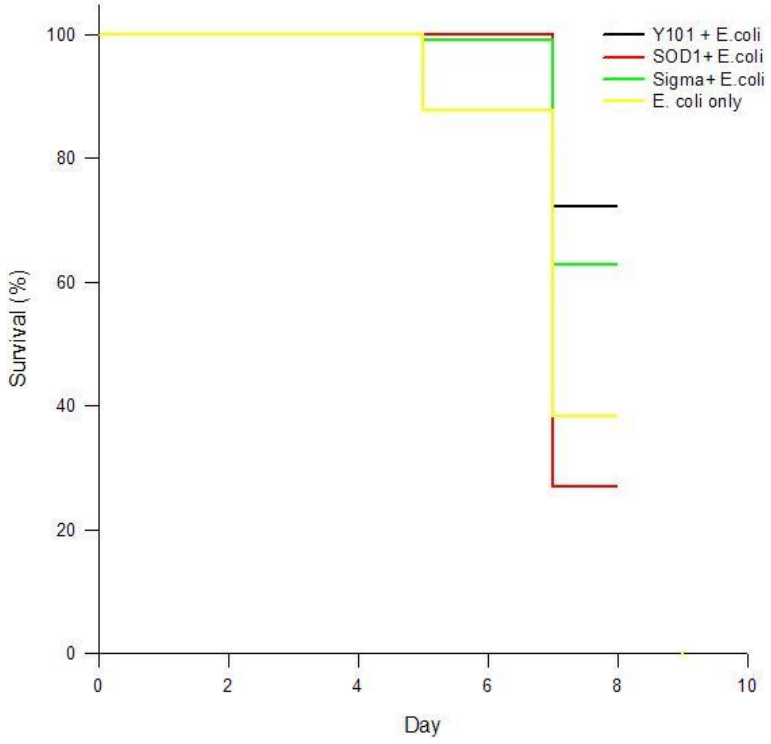


Figure 4: Survival curve of C. elegans with S. cerevisiae mutants and E.coli in NGM

The Y axis is the percentage of live *C.elegans* each day. The X axis is the time of experiment in days. The *C. elegans* were counted every day for 8 days. The mass ratio of *S. cerevisiae: E.coli* was 1:30.

Results of this experiment are shown in Figure 4. *C. elegans* grown on *Y101* and *SOD1* were significantly different (*P=<0.001). Sigma* and *Sod 1Δ* were significantly different from each other (*P=<0.001*). *Sigma* was not significantly different from the *Y101* control (*P=0.141*). However, *C elegans* grown on *Sigma,* which were expected to die faster than *Sod 1Δ,* actually had a higher survival percentage by the end of the experiment. This was probably due to difficulty encountered in counting. *S. cerevisiae* became very dense in the center of wells during experiments, which made the field under the microscope unclear for counting for all the conditions.

Varying Mass Ratios

The mass ratios used for the first experiment were 1:30, 1:60, 1:120, and 1:24 *S. cerevisiae: E. coli.* After this first experiment the media was changed because the *S.cerevisiea* was very thick due to the use of rich medium NGM. S. medium was used subsequently because it contains no carbon or nitrogen source for *S. cerevisiae* to grow and the counting would be more accurate.

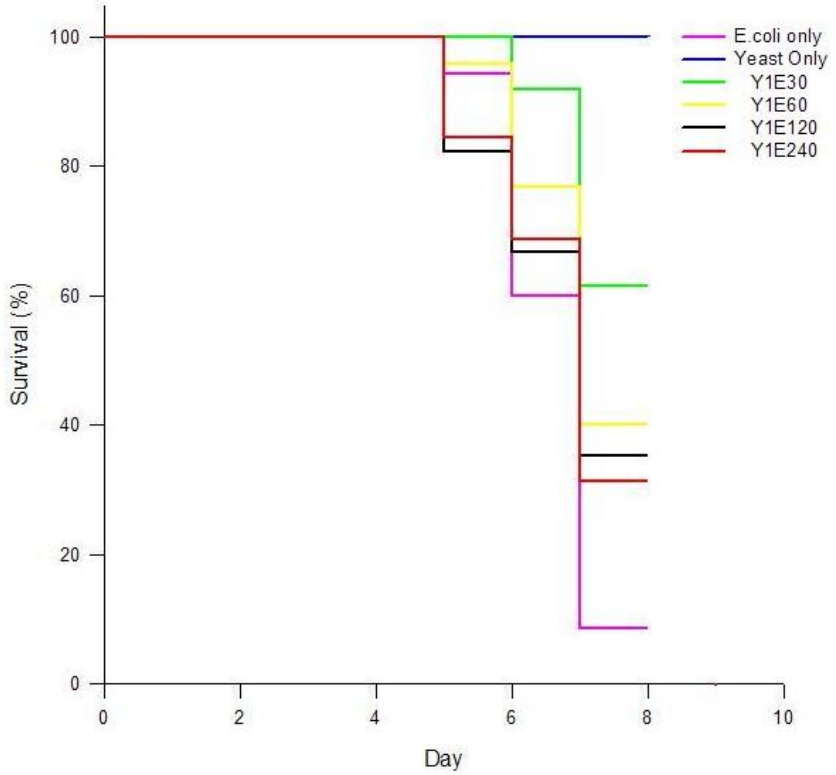


Figure 5: Survival curve of C. elegans with different mass ratio of S. cerevisiae to E. coli in S. medium

Y1E30 is a 1:30 mass ratio of *S. cerevisiae* to *E.coli*. The same notation was used for the other ratios of 1:60, 1:120, and 1:240. The Y axis is the percentage of live *C.elegans* each day. The X axis is the time of experiment in days. The C. elegans were counted every day for 8 days.

Results of an experiment to test the effects of different mass ratios of *S.cerevisiea* and *E.coli* are shown in Figure 5. With a better view of the plate, 1:60, 1:120 and1:240 mass ratios of *S. cerevisiae* to *E.coli* were significantly different from1:30 (*P = <0.001*), using pair wise comparisons. However, the majority of dead *C.elegans* had larva hatched

within their bodies. Whether the *C.elegans* died from *S. cerevisiae* infection or from the hatching larva was unclear.

For the next experiment (Figure 6), *C.elegans* were fed on the third day of the experiment with the same amount of *S. cerevisiae* used to start the experiment. Control wells with *E.coli* only were fed with same amount *E.coli*.

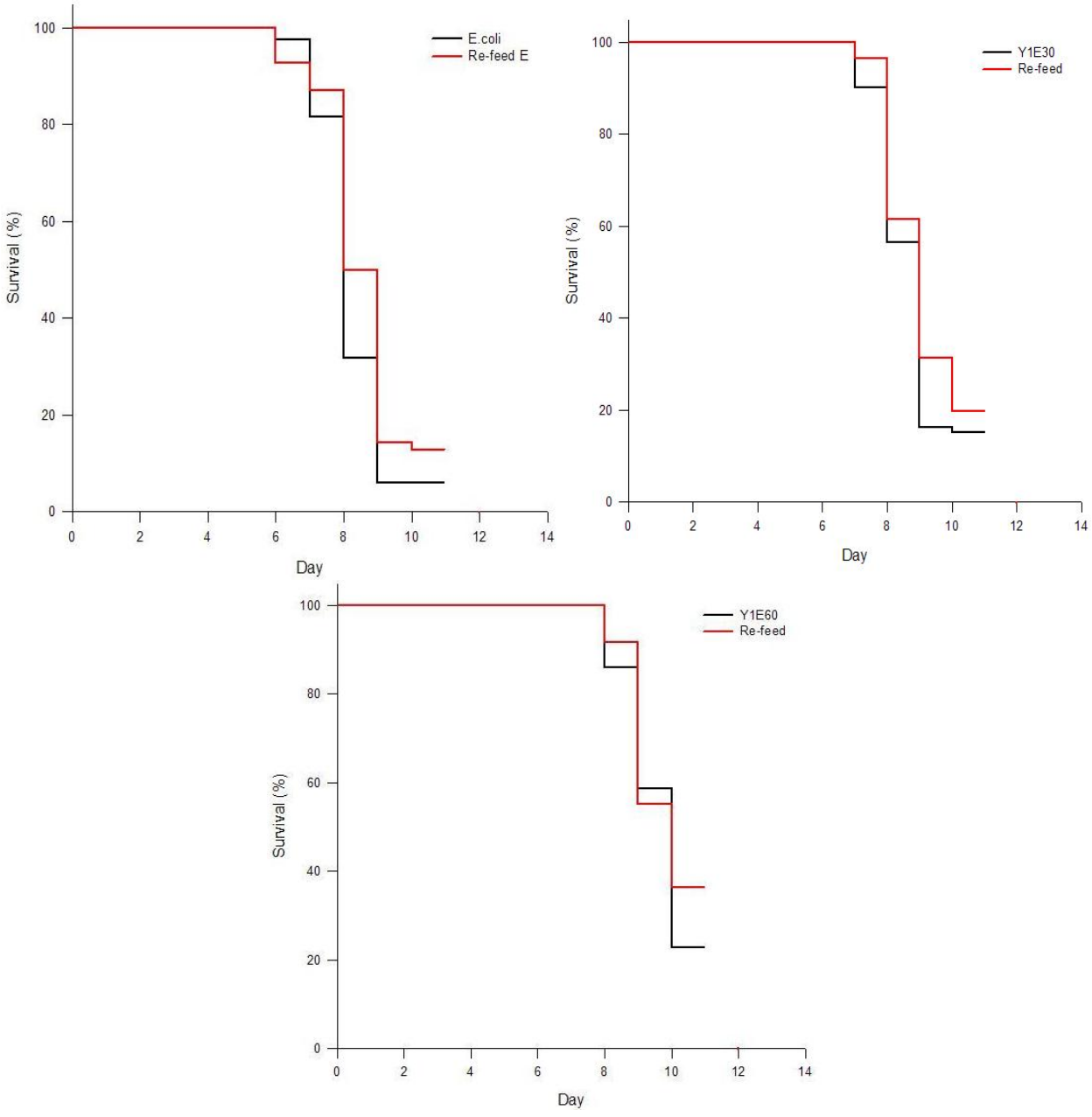


Figure 6: Survival curves of N2 C.elegans at different mass ratios of S. cerevisiae to E. coli with re-feeding

Y1E30 is the mass ratio of *S. cerevisiae* to *E.coli*, 1:30. The same notation is used for 1:60 mass ratios. The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment in days

C.elegans in re-feeding wells did live longer than the non-feeding ones in the *E.coli* only condition (*P = 0.038*). But in 1:30 and 1:60 mass ratios, there was no significant differences between re-feeding and non-feeding conditions (for 1:30, *P = 0.176; for* 1:60, *P=0.134*). However, dead *C.elegans* with larva hatched within bodies were still observed during the experiment.

C. elegans Mutants

The *C. elegans* mutants that were used were *mek-1(ks54)* and *fer-1(b232)*. *Mek-1* encodes a MAP kinase that is required for resistance to killing by *S. cerevisiae* in agar plate cultures (Meijiang Yun, unpublished results).

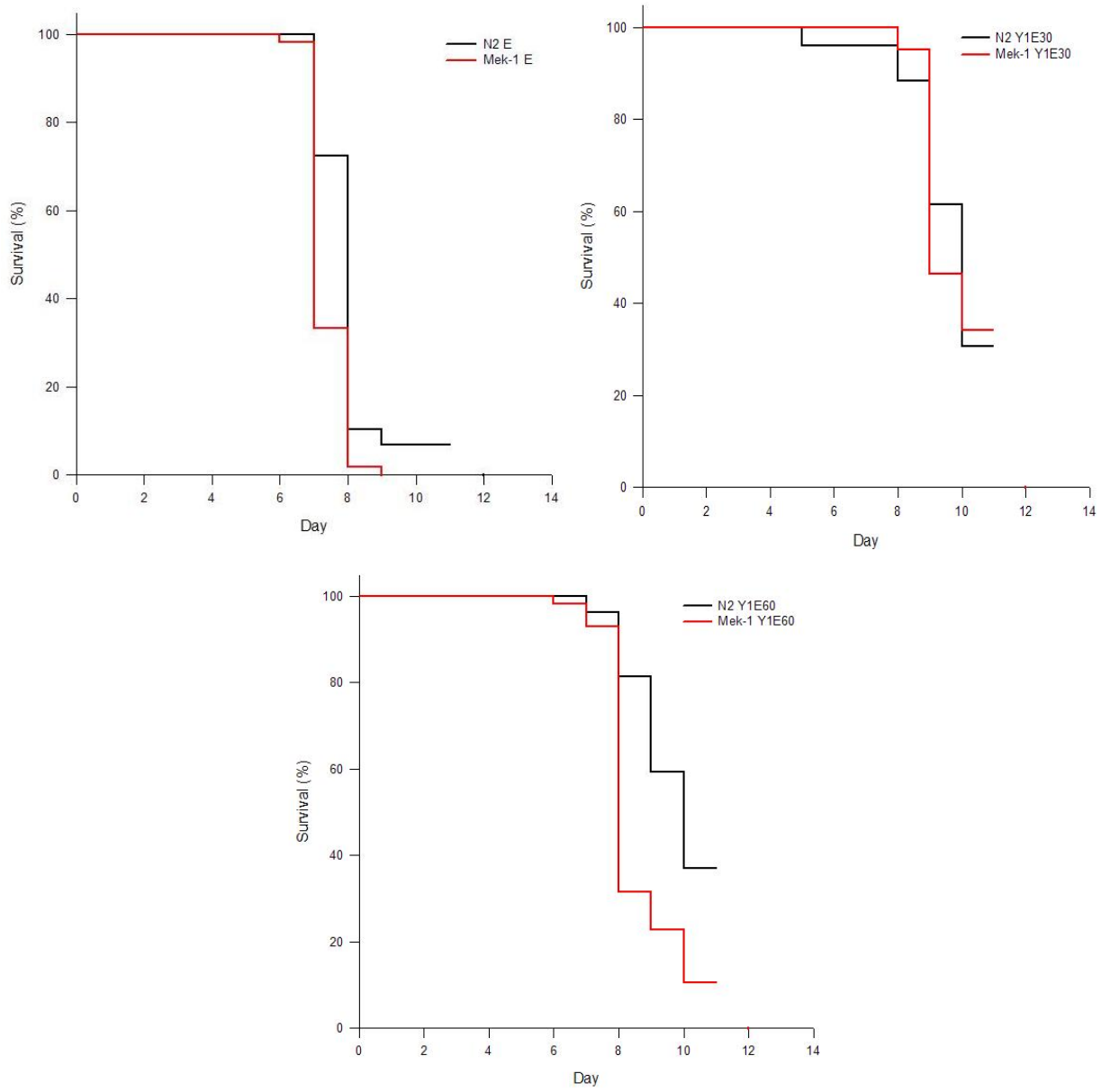


Figure 7: Survival curves of N2 and mek-1 C.elegans at different mass ratios of S. cerevisiae to E.coli with re-feeding

The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment. The number of worms was counted each day for 11 days.

The experiment was done with three different conditions: an amount of equivalent to that mixed with yeast in other samples, a mass ratio of *S. cerevisiae* to *E. coli* of 1:30, and a mass ratio of *S. cerevisiae* to *E. coli* of 1:60. Results are shown in Figure 7. *Mek-1*

died significantly faster than *N2 (P =<0.001)* in both *E.coli* control and 1:60 mass ratio conditions. The P value of 1:30 mass ratio was 0.958, from which we concluded that the 1:30 survival curve is not great enough to exclude the possibility that *mek-1* lived longer than the N2 strand is due to random sampling variability. Both *mek-1* and *N2* had many dead *C.elegans* with larva hatched inside the bodies.

Fer-1 is a *C. elegans* mutant that is temperature-sensitive fertilization defective. At 20 $^{\circ}$C, *fer-1(b232)* hermaphrodites are infertile.

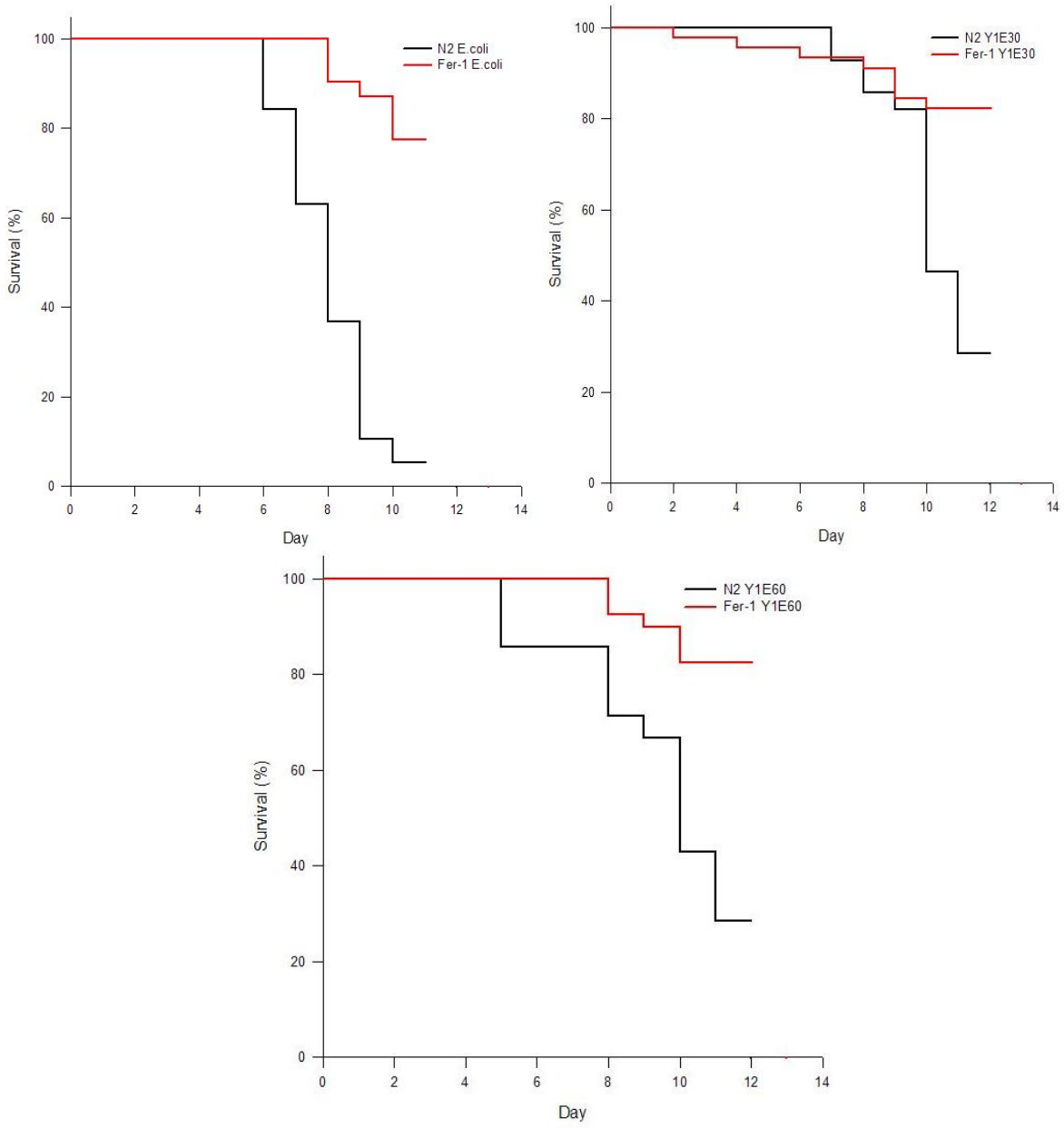


Figure 8: Survival curves of N2 and fer-1 C.elegans at different mass ratios of S. cerevisiae to E.coli at 20°C with re-feeding

The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment in days. The *C. elegans* were counted everyday for 12 days.

The experiment was done with three different conditions: same amount of *E. coli*, a mass ratio of *S. cerevisiae* to *E. coli* of 1:30, and a mass ratio of *S. cerevisiae* to *E. coli* of 1:60. Y1E30 notation means a *S. cerevisiae* to *E. coli* mass ratio of 1:30. The same

notation is used for mass ratio of 1:60. *Fer-1* lived significantly longer than *N2* in the experiment (All curves have a P value of <0.001). About 80% of the *fer-1 C.elegans* lived till the 13^{th} day of the experiment.

Discussion

Using a liquid culture assay would be ideal for identifying fungal virulent factors because it would make the high-throughput screening of the deletion library more efficient. However, the assay has not been fully developed.

When using different mass ratios of *S. cerevisiae* to *E. coli, C. elegans* did not die significantly faster at a lower concentration of *S. cerevisiae* compared to a higher concentration of *S. cerevisiae.* Death could be accounted for fertilized eggs hatching inside the body. While viewing this, there was uncertainty of whether the *C. elegans* died because of *S. cerevisiae* or because of starvation. When the environment is not ideal, *C. elegans* does not lay eggs (Schafer, 2005).

When observing the results obtained with *N2* and *mek-1*, *mek-1* died faster compared to *N2*. There were other factors that influenced the dying of the *C. elegans* such as larva hatching inside of the body. This mutation in the MAP kinase cascade in the immune system may still be useful for high throughput assays because it is weaker and more susceptible to infection.

To eliminate death due to fertilized eggs hatching within the worm's body, we used *fer-1*, a temperature-sensitive fertilization-defective mutant. At the restrictive, 20^{o}C, *fer-1(b232)* sperm are inactivated and eggs consequently are not fertilized. This would prevent the effects of *S. cerevisiae* infection from being obscured and allow the real effects of yeast to be observed. The *fer-1 C. elegans* stayed alive throughout the experiment; only about 20% of the *fer-1 C. elegans* died within 12 days while more than 70% of the *N2* died. The only reason that 100% of the *N2* did not die is that the experiment was terminated at 12 days. This experiment allowed us to conclude that wild type *C.elegans* was not dying due to *S. cerevisiae* but due to the fertilized eggs hatching within the body.

Further Experiment

From the results of experiments using different *C.elegans* mutants, a *fer-1; mek-1* double mutant of *C.elegans* would be a good strain to test for further liquid assay development.

Fer-1 mutant *C. elegans* will not lay any fertilized eggs at 20°C, the temperature used for our liquid assay, which helps exclude the death-causing factor of fertilized eggs hatching inside *C.elegans* bodies from experiment results. Therefore, experiments using *fer-1* mutant *C.elegans* should represent the influence of *Saccharomyce cerevisiae* more accurately.

Mek-1 mutant *C.elegans* is more sensitive to fungal infection due to its mutation in a gene that was previously shown to be important for innate immune responses to bacterial infections (Nicholas and Hodkin, 2004). The effects of fungal infection on *C.elegans* may be more obvious and thus easier to study using this strain of *C.elegans* in liquid assay. The experiment time could also be shortened due to *mek-1*mutant *C.elegans'* sensitivity.

Appendix 1: Raw Data

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
E.coli only	65	86	105	106	98	64	15
Yeast:E.coli 1:30	48	113	112	104	108	100	21
Yeast:E.coli 1:60	68	147	142	141	135	107	48
Yeast:E.coli 1:120	136	151	154	153	125	103	24
Yeast:E.coli 1:240	80	100	95	98	82	67	6

Various Mass Ratios Without Feeding

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
E.coli only	61	81	82	82	82	80	67	26	5	5
E. coli only Fed	60	68	69	69	69	64	60	34	9	8
Yeast:E.coli 1/30	53	87	92	92	92	92	83	52	15	14
Yeast:E.coli 1/30 fed	54	81	86	86	86	86	83	53	27	17
Yeast:E.coli 1/60	86	109	111	114	114	114	114	98	67	26
Yeast:E.coli 1/60 fed	63	96	96	96	96	96	96	88	53	35

Various Mass Ratios With Feeding

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Y101 + E.coli	48	67	101	106	91	68
SOD1+ E.coli	77	99	146	168	192	52
Sigma+ E.coli	39	97	129	116	115	73
E. coli only	37	102	107	128	114	50

Different Yeast Mutants and Stains

N2	day 1	day2	day 3	day4	day5	day6	day7	day8	day9	day10
E.coli re-feed	24	28	28	28	29	29	21	3	2	2
1Y:30E re-feed	19	25	26	26	25	25	25	23	16	8
1Y:60E re-feed	18	25	27	28	28	28	27	23	16	10
Mek-1	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day10
E.coli re-feed	38	49	58	57	57	56	19	1	0	0
1Y:30E re-feed	13	27	35	41	41	41	41	34	19	14
1Y:60E re-feed	28	40	54	54	57	56	53	18	13	6

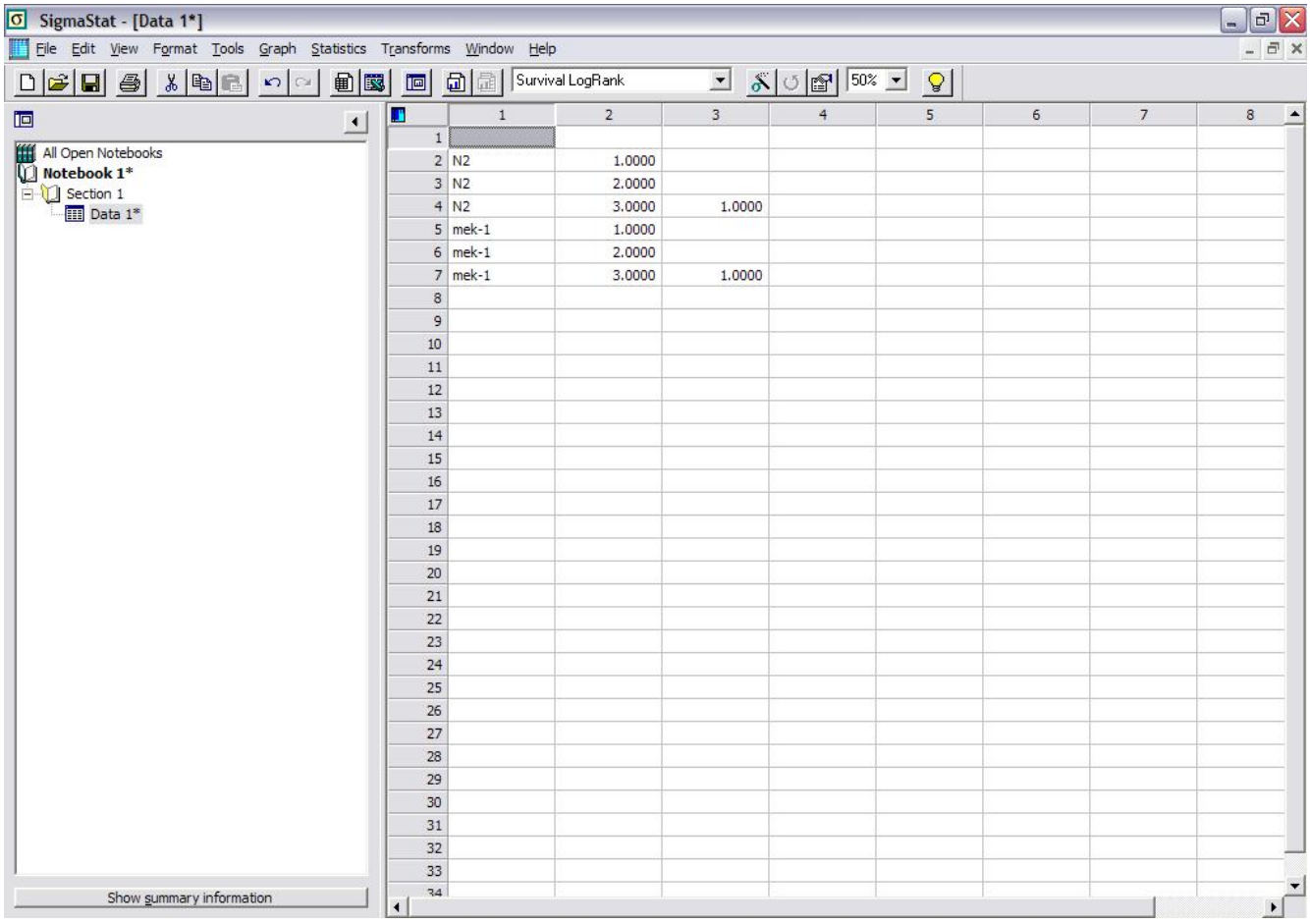
mek-1 and *N2* With Feeding

N2	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day 10	day 11
E.coli re-feed	16	17	19	19	19	16	12	7	2	1	1
1Y:30E re-feed	19	21	28	29	29	29	27	25	23	13	8
1Y:60E re-feed	16	26	21	21	18	18	18	15	14	10	6

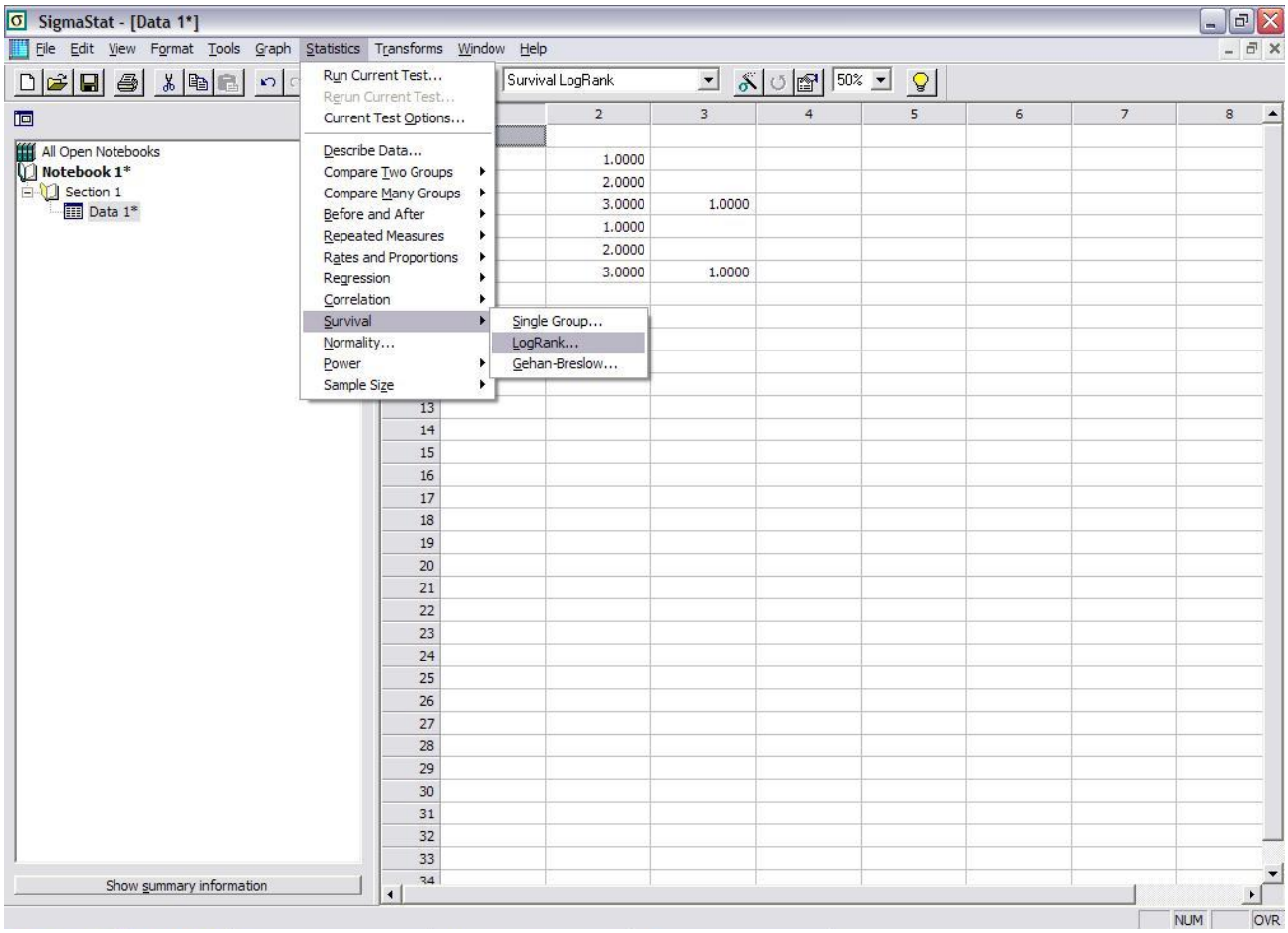
fer-1	day 1	day 2	day 3	day4	day5	day 6	day 7	day8	day9	day10	day 11
E.coli re-feed	23	31	31	31	31	31	31	28	28	24	24
1Y:30E re-feed	15	32	42	42	42	42	42	41	38	37	37
1Y:60E re-feed	23	31	37	37	40	40	40	37	36	33	33

fer-1 and *N2*

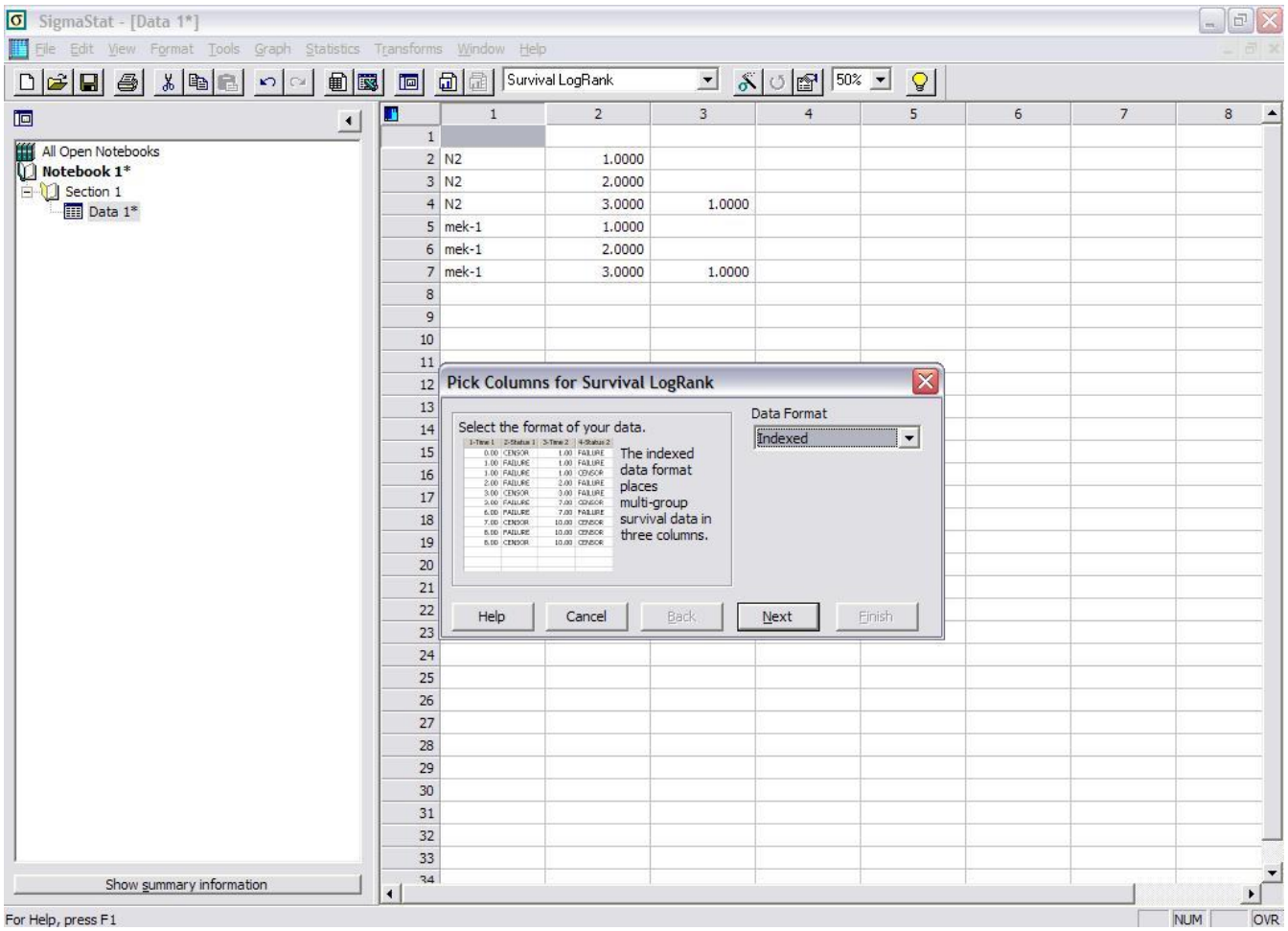
Appendix 2: SigmaStat Set-Up



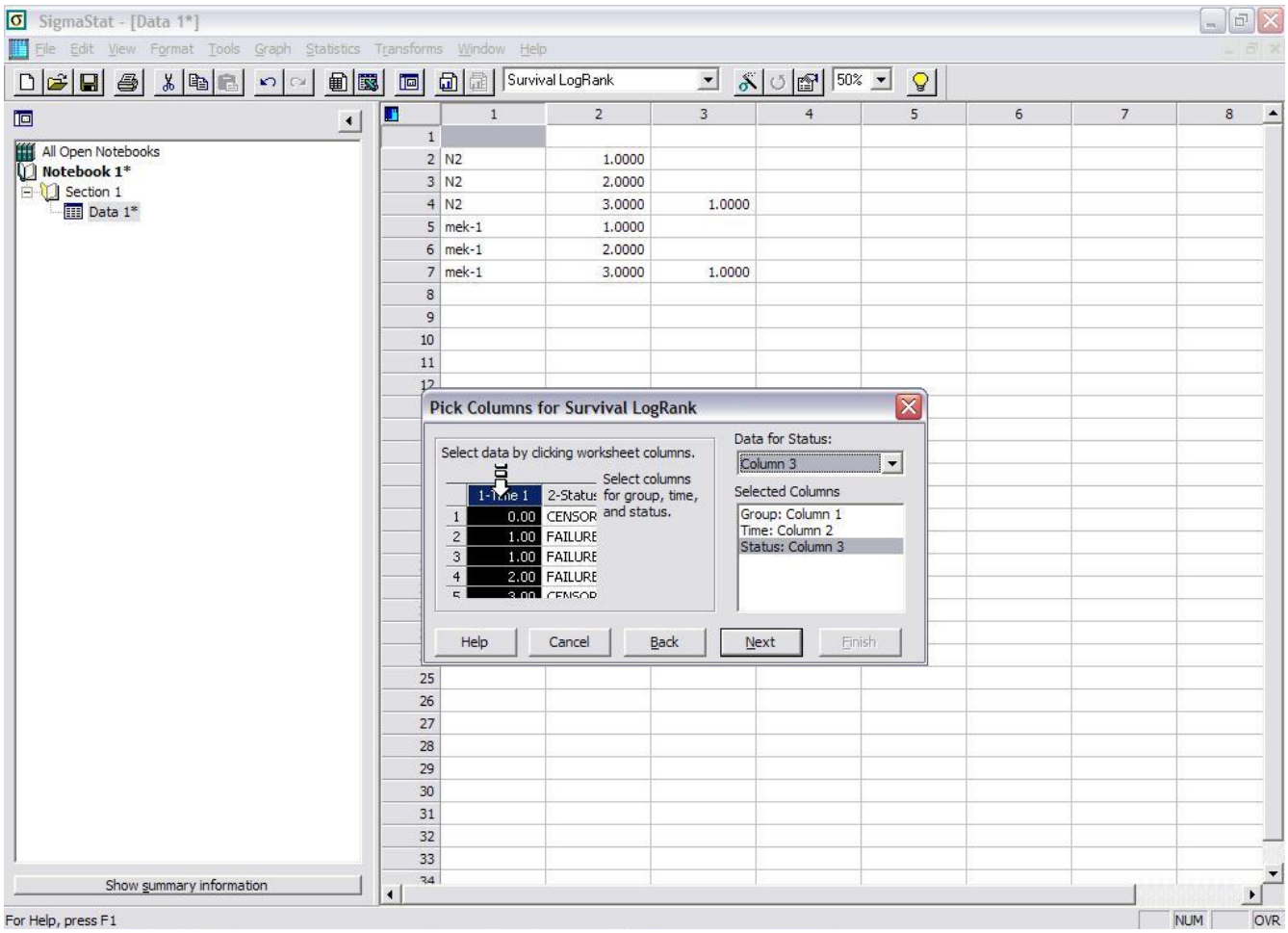
Step 1: Enter Data in Columns



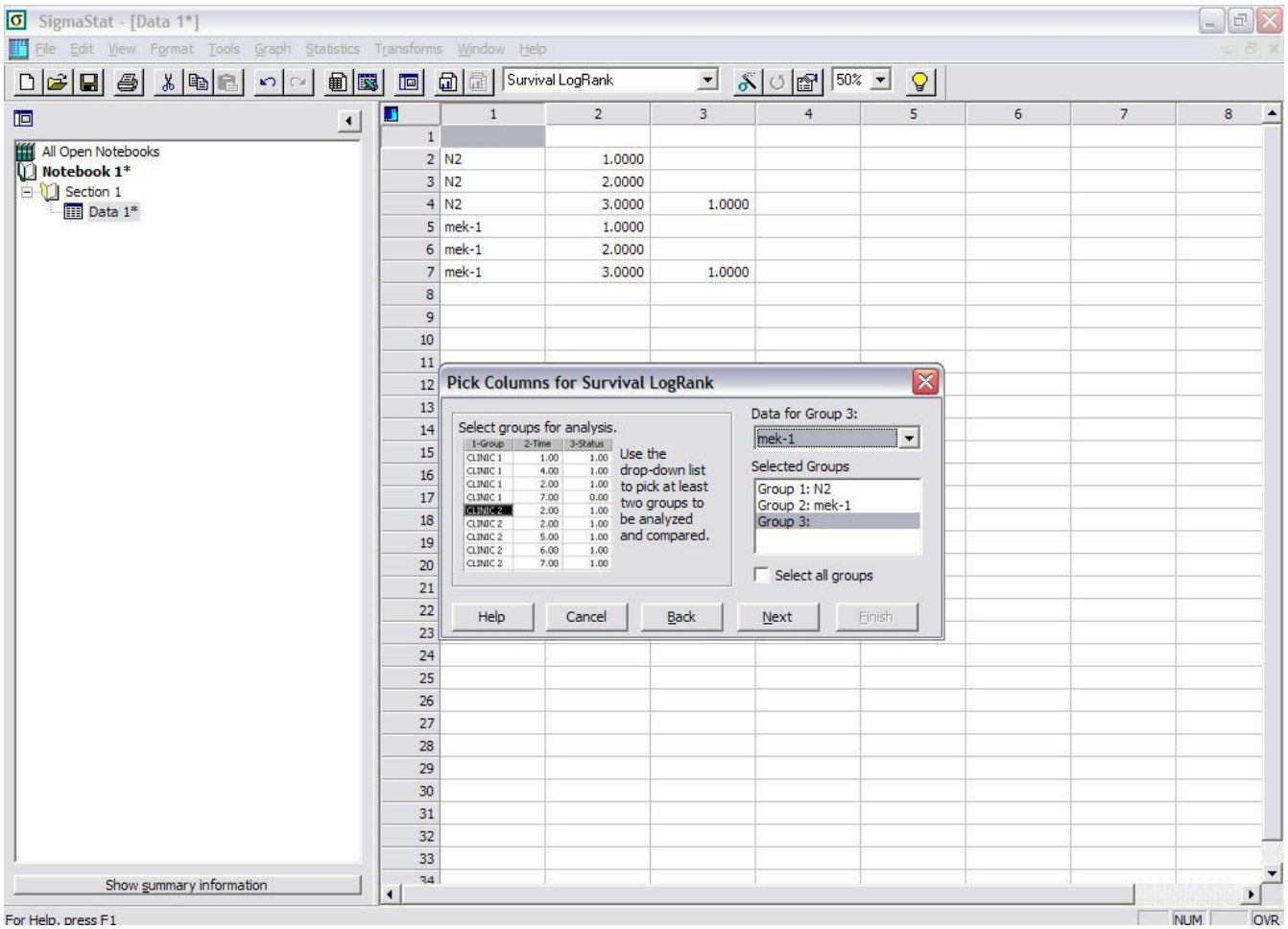
Step 2: Select LogRank Test



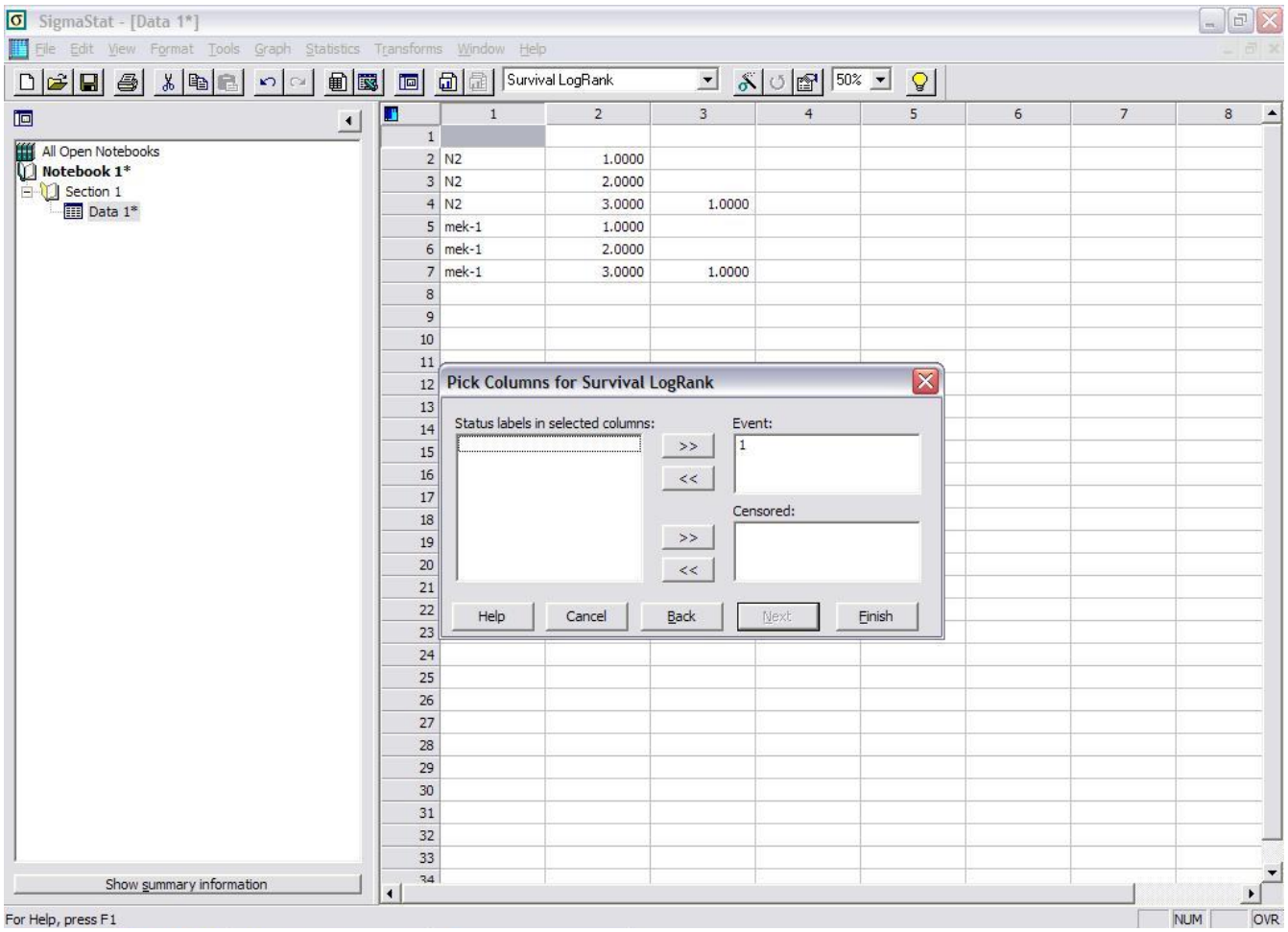
Step 3: Select Data Format



Step 4: Select Group, Time, and Status Columns



Step 5: Select Groups for Comparison



Step 6: Select Event (nothing is censored) and Click Finish

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