Chapter 3

Establishing and validating protocols for high-throughput RNA-mediated interference

3.1. Introduction

RNA-mediated interference (RNAi) is a powerful tool for studying the loss-of-function phenotypes of genes. So far, RNAi has been used extensively to generate genome-scale views of gene functions in the nematode *C. elegans* and in fly cells (for examples see Boutros *et al.*, 2004; Bjorklund *et al.*, 2006; Kamath *et al.*, 2003; Kiger *et al.*, 2003; Simmer *et al.*, 2003). In particular in *C. elegans*, the feasibility of generating loss-of-function phenotypes by feeding worms on bacteria expressing double-stranded RNA (dsRNA) against a target gene of interest ('RNAi by bacterial feeding'; Timmons and Fire, 1998) has led to the generation of a whole-genome RNAi feeding library, consisting of 16,757 dsRNA-expressing bacterial clones corresponding to ~86% of predicted *C. elegans* genes (Kamath *et al.*, 2003). This resource allows for the rapid and low-cost analysis of gene functions on a large scale. Therefore, RNAi by bacterial feeding has become the method of choice for performing genome-scale loss-of-function screens in the worm.

While loss-of-function analyses of individual genes give us an unprecedented level of insight into the molecular roles of genes, genome-wide studies revealed that inactivation of most genes in any organism has little discernible effect on fitness under laboratory conditions (Bjorklund *et al.*, 2006; Boutros *et al.*, 2004; Giaever *et al.*, 2002; Kamath *et al.*, 2003). However, inactivating specific rare combinations of such non-essential genes can have profound effects on the organism under exactly the same conditions, the most dramatic being inviability (Dobzhansky, 1946; Sturtevant, 1956; reviewed in Guarente, 1993; Hartman *et al.*, 2001). These combinatorial effects are termed 'synthetic enhancement' or 'synthetic lethal' interactions. Synthetic lethal (SL) genetic interactions are classically interpreted as the result of inactivating two functionally redundant pathways in the cell, each of which is individually dispensable (reviewed in Guarente, 1993; Hartman *et al.*, 2001). Recently, synthetic sick and synthetic lethal (SL) interactions have been mapped systematically in the yeast *S. cerevisiae*. These large-scale studies appear to have uncovered an extensive degree of redundancy in the genome of *S. cerevisiae*. Strikingly, while only ~1,000 of the ~6,000 genes in the yeast genome are essential for viability under standard laboratory conditions and thus show a lethal phenotype when deleted, systematic large-scale studies have

uncovered thousands of SL interactions under identical conditions. These results led to the estimation that, on a genome-wide scale, inactivation of ~ 200,000 pairwise gene combinations might have detrimental effects (reviewed in Boone *et al.*, 2007). These approximations highlight the complexity of biological functions. Thus, while studying the molecular roles of individual genes is a major advance, an understanding of how each phenotype is modulated by the activities of other genes will prove to be just as critical.

Comparable large-scale approaches to identify SL interactions in more complex systems will reveal the extent of redundancy in different genomes, and will also shed light on the evolution and conservation of gene networks (as discussed in the Introduction).

Extrapolating the estimates from large-scale yeast genetic interaction studies to the genome of *C. elegans*, it is evident that high-throughput platforms are needed for the systematic mapping of genetic interactions in the worm. At the time my study began, RNAi by feeding was conventionally performed on 12-well nematode growth medium (NGM) plates (‘plate feeding’; as discussed in the Introduction, see Figure 1.3). However, the throughput of this approach is limited. It thus was critical to establish protocols that allow RNAi experiments to be performed at considerable higher throughput.

In collaboration with Ben Lehner in the lab, I sought to develop a robust high-throughput (HTP) assay for screening RNAi phenotypes in liquid cultures in 96-well format. There are numerous advantages to performing RNAi screens by liquid feeding in 96-well format over conventional plate-feeding protocols. First, all pipetting steps can be done by using multichannel tools, thereby considerably reducing the time it takes to set up screens. Second, when feeding worms in liquid culture, one can increase the amount of food as compared to plate feeding, thereby allowing multiple worms to be screened in each individual RNAi experiment. Thus, in contrast to screening progeny of individual animals — as is done when using conventional plate-feeding methods — RNAi by liquid feeding would allow the screening of populations of worms for loss-of-function phenotypes. First, this would remove the laborious manual step of transferring single worms into individual wells. Most importantly, however, considering the inherent

animal-to-animal variability in RNAi, screening progeny of individual animals can lead to the observation of rather extreme phenotypes and hence to the representation of greatly biased results. In contrast, analysing the loss-of-function phenotypes of pooled adults allows to identifying the mean phenotype. Thus, screening populations of worms would average the animal-to-animal variation of RNAi phenotypes and lead to more standardized results than obtained when using single-animal plate-feeding protocols. Finally, with the smaller size of 96-well in comparison to 12-well plates, incubation space is unlikely to become a limiting factor for performing RNAi screens on a large scale.

Thus, in summary, RNAi by liquid feeding in 96-well format would allow us to study the loss-of-function phenotypes of entire worm populations, thereby avoiding the animal-to-animal variation of RNAi phenotypes. That way, we would obtain an estimate of the mean RNAi phenotype while substantially increasing the throughput.

In this chapter, I will describe the development of an experimental platform for the screening of RNAi phenotypes in liquid culture in 96-well format and demonstrate that this is an efficient and robust method for analyzing loss-of-function phenotypes in *C. elegans*. Moreover, I will discuss how I have adapted these protocols for using RNAi to simultaneously target two genes in the genome of *C. elegans*.

3.2. Establishing a high-throughput RNAi liquid-feeding assay in 96-well format

To set up an HTP platform for RNA interference (RNAi) by liquid feeding, we considered the following criteria. First, we sought to identify an appropriate volume of liquid to ensure adequate aeration of feeding cultures. Second, we needed to identify the number of larval-stage worms that can be accurately pipetted using a multi-channel pipette while being compatible with the volume of bacterial feeding cultures. Taking into account that flat-bottomed 96-well plates can maximally contain 200 µl per individual well, we reasoned that a total volume of 50 µl might be suitable for liquid cultures to be sufficiently oxygenated without requiring excessive shaking. Next, we sought to determine an adequate number of worms to be dispensed into each well. We wanted to start the 96-well liquid-feeding protocol with first larval stage (L1) worms, because it is

straightforward to obtain synchronized cultures of L1 worms by filtering mixed-stage populations of worms through an 11 µm nylon mesh (MultiScreen^{TM} Nylon Mesh, Millipore), or by bleaching adult worms and allowing their eggs to hatch over night in M9 buffer (see Materials and Methods for a detailed description). We found approximately 10 L1 worms (in a volume of 10 µl) to be the minimum number of animals that can be reproducibly distributed. This low quantity also allows L1s to grow to adulthood, lay eggs and these eggs to hatch without food becoming a limiting factor.

3.2.1. Experimental design

For RNAi experiments, selected bacterial strains of the *C. elegans* RNAi feeding library (Kamath *et al.*, 2003) were inoculated in bacterial culture medium containing ampicillin in deep 96-well plates (see Figure 3.1. for an overview of the experimental procedure and Materials and Methods for a more detailed description of the protocol). Bacterial cultures were grown to saturation while shaking at 37°C before inducing dsRNA expression by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) for 1 hour at 37°C. For RNAi by feeding, we exchanged the bacterial culture medium for Nematode Growth Medium (NGM) supplemented with ampicillin and IPTG. Therefore, bacterial cultures were collected by centrifugation, the supernatant discarded, and pelletet bacteria were resuspended in a volume of NGM equal to bacterial culture medium. Approximately 10 L1 worms were distributed into each well of a 96-well flat-bottom plate and 40 µl of the resuspended bacterial feeding cultures were added. For strains with lower brood size as compared to wild-type worms, roughly 15 L1 worms were used. Plates were incubated shaking at 150 rpm, 20°C, for 4 days. This time period allowed L1 worms to grow to adults, lay eggs and for these eggs to hatch and develop into larvae. After 4 days worms had consumed most of their food, which resulted in clearing of the suspension, thereby allowing easy scoring of phenotypes. Worms were manually assessed for viability, fecundity and growth defects using a dissecting microscope (for example phenotypes see Figure 3.2.). Note that at the same time, others have described similar protocols for performing RNAi screens in 96-well format (Nollen *et al.*, 2004; van Haaften *et al.*, 2004).

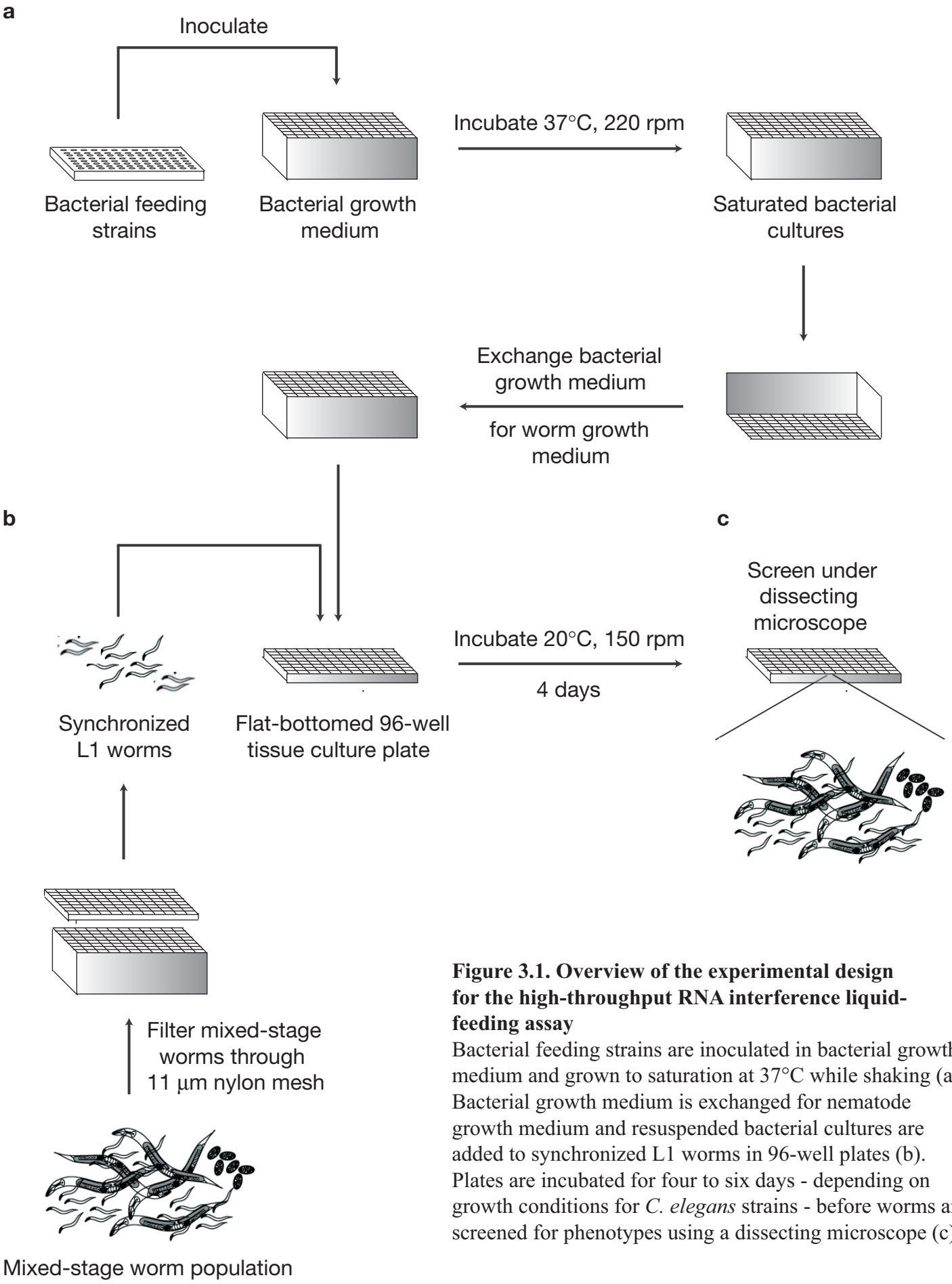

Figure 3.1. Overview of the experimental design for the high-throughput RNA interference liquid-feeding assay

Bacterial feeding strains are inoculated in bacterial growth medium and grown to saturation at 37°C while shaking (a). Bacterial growth medium is exchanged for nematode growth medium and resuspended bacterial cultures are added to synchronized L1 worms in 96-well plates (b). Plates are incubated for four to six days - depending on growth conditions for *C. elegans* strains - before worms are screened for phenotypes using a dissecting microscope (c).

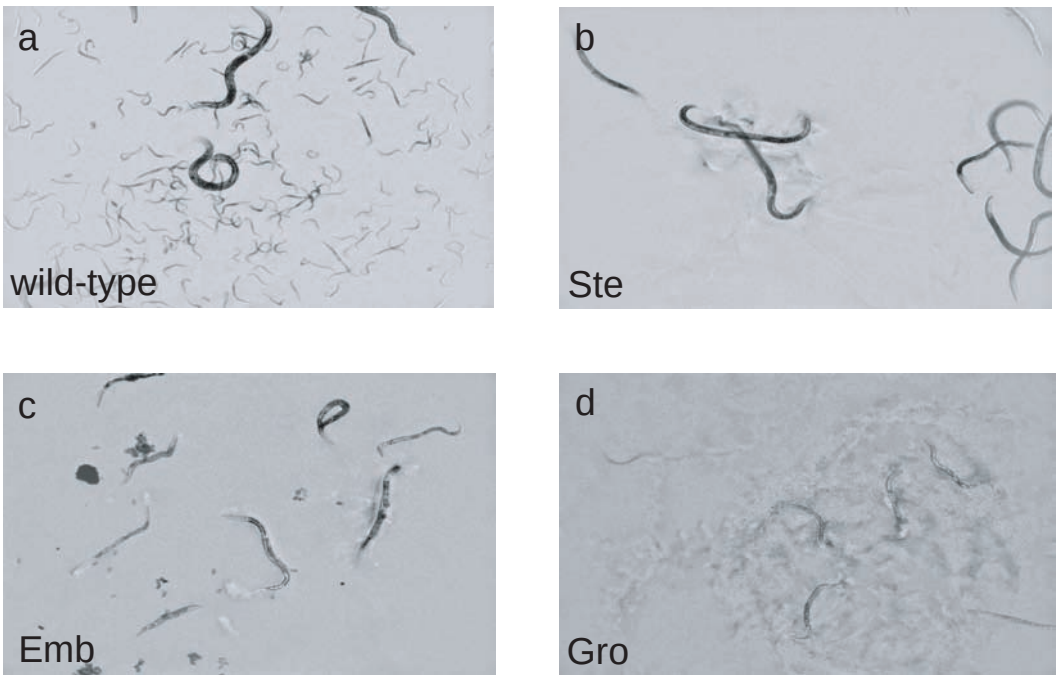

Figure 3.2. Example phenotypes generated by RNA interference by liquid feeding in 96-well format
Representative pictures of wild-type worms (a), sterile (b), embryonic lethal (c, black dots represent dead eggs), and first-generation growth defective (d) worms in 96-well plates are shown.

3.2.2. Determining the sensitivity of RNAi by liquid feeding in 96-well format

To evaluate the sensitivity of RNAi by liquid feeding in 96-well format, I sought to assess the ability of this newly developed assay to recapitulate RNAi phenotypes that have been generated using conventional plate-feeding protocols. Therefore, I selected all 391 genes from *C. elegans* chromosome III that showed an RNAi phenotype in the genome-wide screen performed by Kamath *et al.* (2003) (see Appendix Table 3.1.). I chose to determine the sensitivity of the RNAi liquid-feeding assay in the RNAi-hypersensitive *rrf-3* background, which had previously been found to result in an increased penetrance of RNAi phenotypes as compared to wild-type worms.

First, I tested 282 genes that were reported to give a non-viable (embryonic lethal or sterile) RNAi phenotype and investigated these for non-viable phenotypes in two independent experimental setups. I identified 209 genes (74%) to also result in non-viable RNAi phenotypes in two independent screens by RNAi by liquid feeding. An additional 19 and 10, respectively, genes were found to show non-viable phenotypes in either screen (Table 3.1).

Next, I focused on genes that were known to result in slowed post-embryonic growth. Of 64 genes assayed, 39 genes (61%) gave a detectable RNAi phenotype in two separate screens and a further 9, and 3 genes, respectively, displayed an RNAi phenotype in either screen. The great majority of these genes, however, showed non-viable, rather than slowed growth RNAi phenotypes when screening *rrf-3* worms in my experimental setting (see below for discussion).

Finally, I investigated the detection rate of post-embryonic phenotypes. Of 45 genes with any known post-embryonic RNAi phenotype identified by Kamath *et al.*, 24 genes (53%) showed an RNAi phenotype in both independent screens, and phenotypes were detected for an additional 6, and 4, respectively, genes in either assay. Roughly two thirds of these genes displayed non-viable RNAi phenotypes.

There are two possible explanations for the increased frequency of non-viable RNAi phenotypes that I observed when screening *rrf-3* worms using the 96-well liquid-feeding assay, as compared to the results reported by Kamath and co-workers. First, I used the RNAi-hypersensitive strain *rrf-3*, which is known to result in higher

Kamath *et al.*, 2003 RNAi screen	96 well RNAi liquid feeding assay								
	Detected			Identical			Non-viable		
	Screen 1	Screen 2	Mean	Screen 1	Screen 2	Mean	Screen 1	Screen 2	Mean
Non-viable (n=282)	238 (84%)	232 (82%)	235 (83%)	228 (81%)	219 (78%)	224 (79%)	228 (81%)	219 (78%)	224 (79%)
Growth-defective (n=64)	48 (75%)	42 (66%)	45 (70%)	11 (17%)	8 (13%)	10 (15%)	37 (58%)	33 (52%)	35 (55%)
Post-embryonic (n=45)	30 (67%)	28 (62%)	29 (64%)	4 (9%)	3 (7%)	4 (8%)	20 (44%)	17 (38%)	19 (41%)
Total (n= 391)	316 (81%)	303 (77%)	310 (79%)	243 (62%)	230 (59%)	237 (60%)	285 (73%)	269 (69%)	277 (71%)

Table 3.1. Effectiveness of RNA interference by liquid feeding in 96-well format
RNA interference (RNAi) phenotypes for each gene on *C. elegans* chromosome III that was reported to result in non-viability ('Non-viable'), slowed post-embryonic growth ('Growth-defective') or defects in post-embryonic development ('Post-embryonic') in the genome-wide RNAi screen performed by Kamath *et al*. (2003) were determined when feeding the RNAi-hypersensitive *rrf-3* strain in liquid culture in 96-well format. Worms were assessed for non-viability, slowed post-embryonic growth, and defects in post-embryonic development in two independent experiments ('Screen1', 'Screen 2'). Data shown represent the total number of genes (percentages in brackets) that were detected in each phenotypic category ('Detected'), that had phenotypes identical to the ones reported by Kamath *et al.* ('Identical'), and that resulted in non-viability ('Non-viable') after RNAi, respectively. Mean values for both independent screens are rounded up.

penetrance of RNAi phenotypes as compared to wild-type worms, while Kamath *et al.* have performed their genome-wide screen in wild-type animals. Second, I delivered dsRNA to L1 worms, whereas protocols for RNAi by plate feeding start with L3-stage worms. This early interference of gene expression is likely to lead to more severe developmental defects. When comparing my results to previously reported RNAi phenotypes (www.wormbase.org), I found that the great majority (87%) of genes that resulted in non-viability rather than in slowed post-embryonic growth or other post-embryonic defects — as found by Kamath *et al.* (2003) — were shown to have a non-viable phenotype in other RNAi screens, demonstrating that my results are not false positives.

In summary, RNAi by liquid feeding in 96-well format using the RNAi-hypersensitive *rrf-3* strain allowed, on average, the detection of approximately 80% of genes with a previously identified RNAi phenotype in wild-type worms when using conventional plate-feeding protocols. I was able to re-discover over 80% of previously known non-viable RNAi phenotypes. In addition, approximately 70% of genes conferring growth defects and two thirds of genes resulting in any visible post-embryonic phenotype upon RNAi in wild-type worms were identified by bacterial feeding in liquid culture with roughly 90% reproducibility. Importantly, even when only scoring for non-viable phenotypes using the RNAi-hypersensitive *rrf-3* strain, this HTP RNAi liquid-feeding assay can, on average, capture approximately 60% of any phenotypes that have been detected in wild-type worms when using conventional plate-feeding protocols.

Taken together, these results demonstrate that RNAi by bacterial feeding in liquid culture in 96-well format is a powerful tool for generating loss-of-function phenotypes for *C. elegans* on a large scale. Thus, although RNAi phenotypes for some genes might be missed in this assay, RNAi by liquid feeding in 96-well format makes it feasible to perform roughly two thousand individual RNAi experiments per researcher per day, thereby increasing the throughput as compared to conventional plate-feeding protocols by approximately ten-fold. Most notably, by pooling animals, one can average the animal-to-animal variation of RNAi phenotypes. To obtain similarly standardized results by RNAi by plate feeding, phenotypes for numerous individual adults and their progeny would

need to be assessed. In that respect, the throughput of RNAi by liquid feeding is considerably higher than the estimated 10-fold, but rather ranges around 50-fold.

In summary, I consider the RNAi liquid-feeding assay in 96-well format using the RNAi-hypersensitive *rrf-3* background a HTP screening platform and an efficient and robust method for generating genome-scale views of gene function in *C. elegans*. I will refer to this screening tool as the 'high-throughput (HTP) RNAi liquid-feeding assay' throughout my text.

3.2.3. Targeting multiple genes simultaneously by combinatorial RNAi

While analyzing the loss-of-function phenotypes of individual genes in an essentially wild-type background is a major advance, understanding how each phenotype is modulated by the activities of other genes will give us deeper insights into the complexity of biological functions. If mutations in one gene modulate the mutant phenotype of a second gene, these two genes are said to genetically interact (discussed in Hartman *et al.*, 2001).

To identify genetic interactions and to uncover genetic redundancy systematically in *C. elegans*, it was critical to establish methods for simultaneously perturbing two genes. Therefore, the concurrent targeting of two genes by RNAi would provide a powerful approach for unbiased searches for genetic interactions in the worm. Previously, it had been shown that injection of two dsRNAs targeting two individual loci could effectively reduce the expression of both genes simultaneously (Gotta and Ahringer, 2001; Paradis and Ruvkun, 1998; Pocock *et al.*, 2004). However, because RNAi by injection is both very labour-intensive and costly, its applications are limited. In contrast, targeting two genes simultaneously by RNAi by bacterial feeding would allow the systematic study of bigenic interactions in the genome of *C. elegans*.

Thus, to be able to carry out unbiased screens for genetic interactions in *C. elegans*, I sought to establish conditions for simultaneously targeting two genes using RNAi by bacterial feeding. Therefore, I considered three potential approaches (Figure 3.3.). One possibility would be the generation of a 'two-gene' bacterial feeding library by

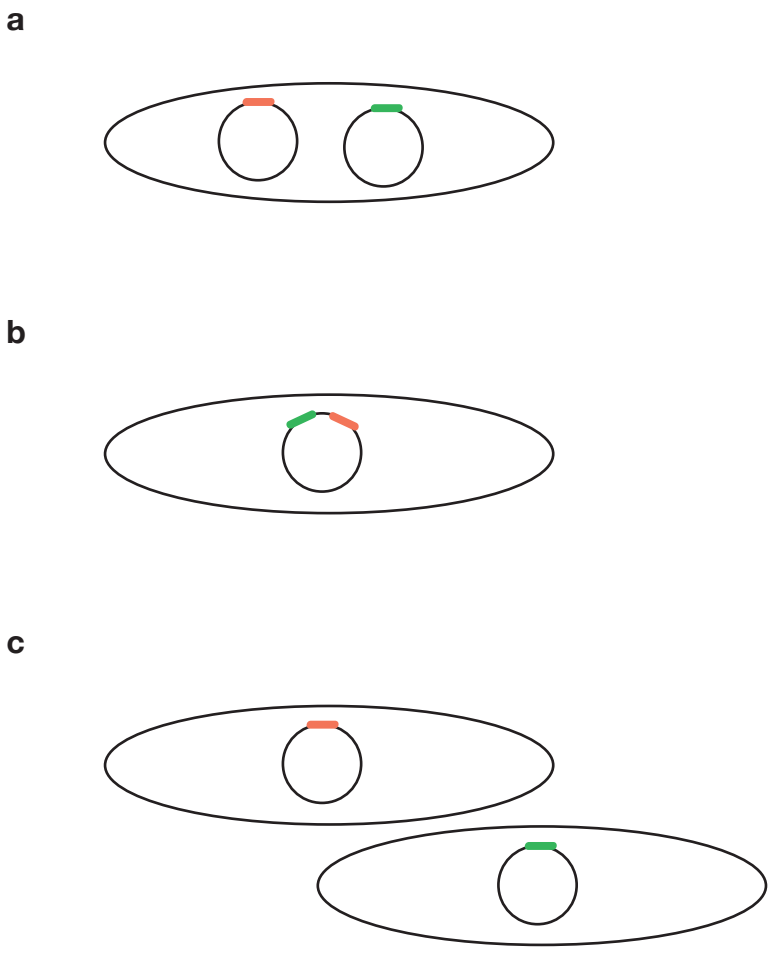

Figure 3.3. Three approaches for using RNA interference by bacterial feeding to simultaneously target two genes
Bacterial strains are transformed with two 'RNA interference (RNAi) feeding vectors' (L4440), each engineered to expressing a dsRNA against a different target gene of interest (a). Bacterial strains are transformed with one RNAi feeding vector, engineered to express two dsRNAs, each targeting a different gene of interest (b). Mixing of two RNAi feeding clones, each expressing a dsRNA targeting a different gene of interest (c). Ellipses, bacterial feeding strains. Circles, RNAi feeding vectors. Red and green rectangles, DNA fragments complementary to the coding regions of two different genes of interest.

co-transforming two ‘RNAi feeding vectors’ (L4440), each harbouring a different selection marker and engineered to express dsRNA against any target gene of interest, into one bacterial feeding strain. Alternatively, one might insert two DNA fragments complementary to the coding regions of any two genes of interest into the same RNAi feeding vector. This approach has previously been reported to result in the concomitant knockdown of two genes (P. Kuwabara, personal communication). However, taking into account that this method would require the laborious cloning of all pairwise combinations of gene-specific DNA fragments, this strategy might not be suitable for an exhaustive screening of bigenic interactions in *C. elegans*. Conversely, the most direct approach would be the feeding of worms on a mixture of two dsRNA-expressing bacterial strains. In principle, this would allow the systematic examination of interactions between any pair of genes on a large scale. Previous studies aimed at the simultaneous targeting of two genes by feeding worms on two different dsRNA-expressing bacteria, however, reported a reduced strength of phenotype produced by either gene (A.G. Fraser and R. Kamath, personal communication). However, these anecdotal negative results have come from a small number of experiments performed on wild-type worms. Given the potential power of this approach for the comprehensive mapping of genetic interactions in the genome of *C. elegans*, I sought to carefully assess the effectiveness of this strategy again, using the RNAi-hypersensitive *rrf-3* background. Therefore, I wished to adapt the high-throughput (HTP) RNAi liquid-feeding assay, which is very efficient and robust for studying the loss-of-function phenotypes of single genes, to targeting two genes simultaneously by mixing two dsRNA-expressing bacterial strains. I will refer to this method as ‘combinatorial RNAi by bacterial feeding’ or simply as ‘combinatorial RNAi’.

3.2.3.1. Testing additive RNAi phenotypes and known synthetic genetic interactions

To investigate whether I could target effectively more than one gene in a single animal by feeding a mixture of two different dsRNA-expressing bacterial strains, I performed three sets of test experiments. In each test set, I sought to determine the effectiveness of combinatorial RNAi both in wild-type worms and in the RNAi-hypersensitive strain *rrf-3*, using the HTP RNAi liquid-feeding assay. First, I sought to

assess whether I could simultaneously target two independent genes, each with a known loss-of-function phenotype, and generate phenotypes for both genes in the same animal — for example, targeting *lin-31* by RNAi generates multivulval worms, targeting *sma-4* results in small worms, and targeting both would be expected to generate small worms with multiple vulvae if combinatorial RNAi was effective. Second, I wished to test whether I can recapitulate genetic interactions between the well-studied 'synthetic multivulval' (synMuv) genes, which have roles in two functionally redundant pathways (see page 61 for a more detailed description). Finally, I sought to investigate whether I can detect a subset of previously described synthetic lethal (SL) interactions.

For combinatorial RNAi feeding experiments, individual clones from the *C. elegans* RNAi feeding library were grown as described above (see Materials and Methods for more detail). After having dispensed first larval stage (L1) worms into each well of a flat-bottomed 96-well plate, equal volumes of two different bacterial feeding cultures were added. To be able to screen for second-generation post-embryonic phenotypes — as I intended to do when screening for additive and synMuv phenotypes — second-generation L1 worms were collected from the 96-well liquid-feeding assay by filtration through an 11 μm nylon mesh (MultiScreen^{TM} Nylon Mesh, Millipore), and allowed to develop further on 12-well Nematode Growth Medium (NGM) plates seeded with bacteria expressing a dsRNA that does not target a transcribed locus of the *C. elegans* genome (Ahringer RNAi feeding library clone Y95B8A_84.g; Figure 3.4.). Growing second-generation L1s to adults on NGM plates by feeding them on non-targeting — as compared to targeting — dsRNA-expressing bacteria ensured that the observed post-embryonic phenotypes were generated by using the HTP RNAi liquid-feeding assay and were not caused by RNAi by plate feeding.

3.2.3.1.1. Generating additive phenotypes by combinatorial RNAi

To examine whether I could generate loss-of-function phenotypes for two genes in the same animal by using combinatorial RNAi, I chose four well-characterized genes with non-overlapping post-embryonic phenotypes (Table 3.2.) to ensure that I could investigate each phenotype independently. Examining all possible pairwise combinations

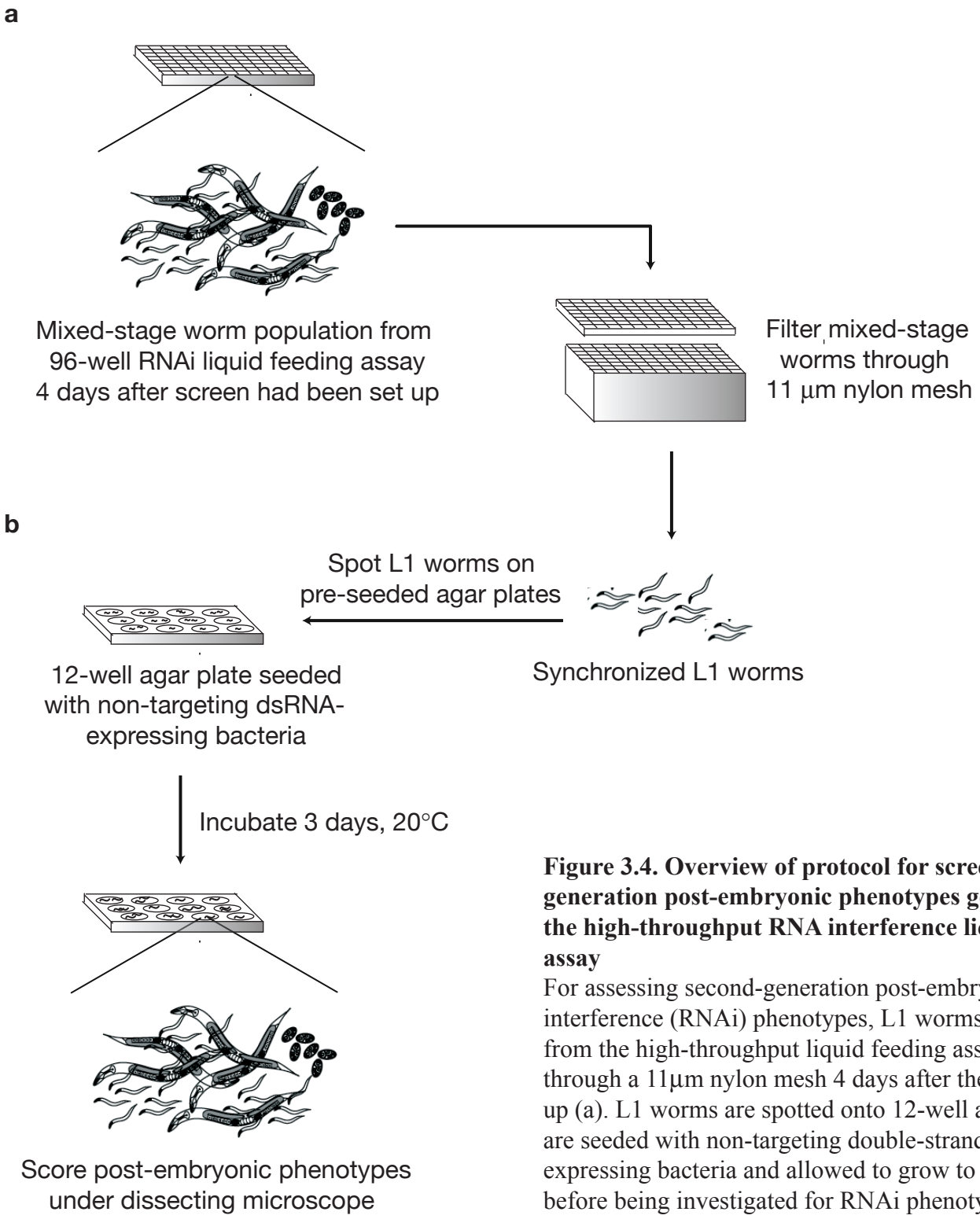

Figure 3.4. Overview of protocol for screening second-generation post-embryonic phenotypes generated in the high-throughput RNA interference liquid-feeding assay

For assessing second-generation post-embryonic RNA interference (RNAi) phenotypes, L1 worms are collected from the high-throughput liquid feeding assay by filtration through a 11µm nylon mesh 4 days after the screen was set up (a). L1 worms are spotted onto 12-well agar plates that are seeded with non-targeting double-stranded RNA-expressing bacteria and allowed to grow to adulthood before being investigated for RNAi phenotypes (b).

Gene1	Gene2	Wild-type		*rrf-3*	
		Pheno Gene1	Pheno Gene2	Pheno Gene1	Pheno Gene2
lin-31	-	5%	-	35%	-
sma-4	-	100%	-	100%	-
unc-22	-	100%	-	100%	-
lon-2	-	100%	-	100%	-
lin-31	*sma-4*	2%	100%	20%	100%
lin-31	*unc-22*	2%	100%	26%	100%
lin-31	*lon-2*	4%	100%	13%	100%
sma-4	*unc-22*	100%	100%	100%	100%
sma-4	*lon-2*	100%	0%	100%	0%
unc-22	*lon-2*	100%	100%	100%	100%

Table 3.2. Combinatorial RNA interference effectively generates additive phenotypes
Wild-type and RNA interference- (RNAi-) hypersensitive *rrf-3* worms, respectively, were fed on selected bacterial strains of the *C. elegans* RNAi feeding library targeting the genes *lin-31*, *sma-4*, *unc-22*, and *lon-2*. Independent RNAi phenotypes ('Pheno Gene1', 'Pheno Gene2') were assessed when each gene was targeted individually and also for all possible pairwise combinations of genes. Percentages represent penetrance of phenotypes.

of my set of test genes and scoring for the known RNAi phenotypes both in wild-type animals and in the RNAi-hypersensitive *rrf-3* background, I could detect five of the five possible additive phenotypes in both wild-type and *rrf-3* worms (Table 3.2.; see Figure 3.5. for an example), demonstrating that it is feasible to target two genes in the same animal by combinatorial RNAi by bacterial feeding. In addition to generating additive phenotypes, I found that the simultaneous targeting of *sma-4* and *lon-2* produced only small worms — the phenotype of *sma-4* alone. Thus, using combinatorial RNAi, it was also possible to recapitulate a previously demonstrated epistatic relationship between SMADs and *lon-2* (Brenner, 1974). Finally, while I could detect additive RNAi phenotypes in wild-type worms, I noted that the penetrance was often higher in the *rrf-3* RNAi-hypersensitive strain, suggesting that this background might be more suitable for combinatorial RNAi. I examine this in more detail below.

3.2.3.1.2. Creating synthetic post-embryonic phenotypes by cmbinatorial RNAi

Next, I investigated whether I could use combinatorial RNAi to recapitulate known genetic interactions that resulted in post-embryonic phenotypes. Therefore, I focused on the well-characterized synthetic multivulval (synMuv) genes (Ferguson and Horvitz, 1989; Poulin *et al.*, 2005). The synMuv genes are organized into two redundant genetic pathways that are required for normal development of the hermaphrodite vulva. Inactivation of either a gene functioning in the synMuv A pathway or a gene functioning in the synMuv B pathway alone does not result in a vulval defect, but inactivation of both a synMuv A and a synMuv B gene in combination leads to the generation of multiple vulvae — the multivulva (Muv) phenotype.

I set out to use combinatorial RNAi to co-target previously identified synMuv A genes (Poulin *et al.*, 2005) with the canonical class B gene *lin-15B*, and synMuv B genes (Poulin *et al.*, 2005) with the canonical synMuv A gene *lin-15A* in both wild-type animals and in the RNAi-hypersensitive strain *rrf-3*, respectively (Table 3.3.). As control, I studied the loss-of-function phenotypes of all synMuv genes individually. In each experiment, I scored progeny for the Muv phenotype; I expected to see this phenotype only if combinatorial RNAi targeted both genes effectively in the same animal. Because I

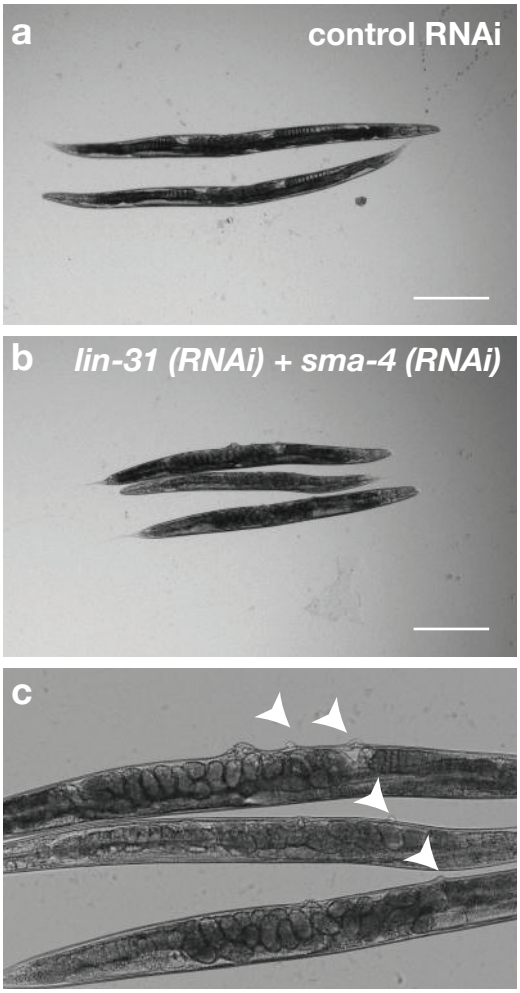

Figure 3.5. Combinatorial RNA interference can target two genes in the same animal
Exposing worms to a mixture of two double-stranded RNA- (dsRNA-) expressing bacterial clones, one targeting *lin-31*, the other one targeting *sma-4*, resulted in small worms with multiple vulvae along their ventral side. Shown are *rrf-3* animals fed on bacteria expressing a non-targeting dsRNA (control, a) and combined bacterial clones expressing dsRNA against *lin-31* and *sma-4* (b and magnified in c). White arrowheads indicate pseudovulvae. Scale bars: 0.1 mm.

lin-15B

Predicted Gene	**Locus**	**synMuv**	**Wild-type**	***rrf-3***
T27C4.4	*egr-1*	A	-	-
ZK678.1	*lin-15A*	A	**Muv**	**Muv**
K12C11.2	*smo-1*	A, B	n.s.	n.s.
W02A11.4	*uba-2*	A, B	**Muv**	**Muv**

lin-15A

Predicted Gene	**Locus**	**synMuv**	**Wild-type**	***rrf-3***
K12C11.2	*smo-1*	A, B	n.s.	n.s.
W02A11.4	*uba-2*	A, B	-	**Muv**
C32F10.2	*lin-35*	B	**Muv**	**Muv**
C47D12.1	*trr-1*	B	n.s.	n.s.
C53A5.3	*hda-1/gon-10*	B	n.s.	n.s.
E01A2.4		B	-	-
F44B9.6	*lin-36*	B	-	**Muv**
JC8.6		B	n.s.	n.s.
K07A1.12	*lin-53/rba-2*	B	n.s.	n.s.
M04B2.1	*mep-1/gei-2*	B	-	**Muv**
R05D3.11	*met-2*	B	-	**Muv**
R06C7.7	*rls-1/lin-61*	B	**Muv**	**Muv**
W01G7.3		B	n.s.	n.s.
W07B3.2	*gei-4*	B	n.s.	n.s.
Y71G12B.9		B	-	**Muv**
Y1O2A5C.18	*efl-1*	B	**Muv**	**Muv**
ZK632.13	*lin-52*	B	**Muv**	**Muv**
ZK637.7	*lin-9*	B	**Muv**	**Muv**
ZK662.4	*lin-15B*	B	**Muv**	**Muv**

Table 3.3. Genetic interactions of synthetic multivulval genes can be recapitulated by combinatorial RNA interference

Previously studied synthetic multivulval (synMuv) genes were targeted by combinatorial RNA interference (RNAi) in wild-type and *rrf-3* worms, respectively. Predicted gene names, their corresponding genetic locus names (if applicable), a definition of the gene as a component of either the synMuv A ('A'), synMuv B ('B'), or both ('A, B') pathways are shown. All synMuv A genes were targeted by RNAi in combination with a double-stranded RNA- (dsRNA-) expressing strain targeting the synMuv B gene *lin-15B*; corresponding experiments were performed with synMuv B genes and a dsRNA-expressing strain targeting *lin-15A*. In both cases, worms were scored for the presence of

was assessing second-generation post-embryonic phenotypes, I had to exclude genes that resulted in sterility, embryonic lethality, or larval growth arrest after RNAi from the screen for synMuv animals (marked as 'n.s.' in Table 3.3.). Of 3 synMuv A genes and 12 synMuv B genes that were amenable to analysis by combinatorial RNAi in both wild-type worms and the RNAi-hypersensitive *rrf-3* background, I observed Muv worms for 13 of 15 test cases in the hypersensitive *rrf-3* background, and for 8 of 15 possible viable combinations in wild-type animals (Table 3.3.).

3.2.3.1.3. Generating known synthetic lethal phenotypes by combinatorial RNAi

As a final test of the efficacy of combinatorial RNAi, I investigated whether I can use combinatorial RNAi to recapitulate a set of known synthetic lethal (SL) interactions compiled from literature (Baugh *et al.*, 2005; Davies *et al.*, 1999; Pocock *et al.*, 2004; Solari *et al.*, 1999; Zhang and Emmons, 2001; Table 3.4.). To do so, I set out to compare the RNAi phenotypes resulting from simultaneously targeting both genes of a SL pair by combinatorial RNAi with the RNAi phenotypes of each gene alone, both in wild-type animals and in the RNAi-hypersensitive strain *rrf-3*, respectively, using the HTP RNAi liquid-feeding assay. To control for mixing two dsRNA-expressing strains when targeting both genes of a pair, I added equal amounts of bacteria expressing a dsRNA that does not target a transcribed portion of the *C. elegans* genome (Ahringer RNAi feeding library clone Y95B8A_84.g) to bacteria expressing dsRNA targeting each gene of a SL pair alone. In order to unambiguously identify SL interactions, I sought to quantify brood sizes per individual adult and embryonic survival rates and assessed these quantitative phenotype data under a multiplicative model (as discussed in the Introduction). Brood size, and embryonic survival rates, respectively, resulting from simultaneously targeting two genes had to be significantly lower than the calculated product of values for both individual genes for a gene pair to be considered SL.

Thus, I first determined brood sizes per individual adult and embryonic survival rates, respectively, following combinatorial RNAi against both genes simultaneously and RNAi against each gene individually in two separate experiments. I manually counted

	Wild-type								
Interaction Gene1 & Gene2	**Gene1**		**Gene2**		**Gene1 & 2**		**SL**	***p*-value**	***p*-value**
	BS	**ES**	**BS**	**ES**	**BS**	**ES**		**BS**	**ES**
mec-8 + sym-1	88	99	82	98	78	92	yes	5.5E-01	1.3E-02
sop-3 + sop-1	91	100	94	99	79	90	yes	2.8E-01	8.5E-04
tbx-8 + tbx-9	83	99	78	97	52	11	yes	7.3E-02	1.4E-24
hlh-1 + unc-120	91	99	76	99	28	91	yes	5.2E-05	1.2E-02
hlh-1 + hnd-1	88	97	75	98	62	81	yes	6.6E-01	5.7E-03
unc-120 + hnd-1	54	100	74	98	36	100	no	6.4E-01	1.9E-01
egl-27 + egr-1	93	99	79	90	90	89	no	6.0E-02	7.4E-01

	rrf-3								
Interaction Gene1 & Gene2	**Gene1**		**Gene2**		**Gene1 & 2**		**SL**	***p*-value**	***p*-value**
	BS	**ES**	**BS**	**ES**	**BS**	**ES**		**BS**	**ES**
mec-8 + sym-1	67	73	61	73	59	16	yes	3.3E-01	3.0E-06
sop-3 + sop-1	82	100	85	96	41	75	yes	3.1E-04	5.7E-06
tbx-8 + tbx-9	96	99	86	92	59	2	yes	8.6E-03	6.3E-27
hlh-1 + unc-120	90	90	31	99	1	64	yes	8.1E-06	2.9E-03
hlh-1 + hnd-1	86	87	82	94	42	24	yes	1.6E-03	8.2E-14
unc-120 + hnd-1	33	100	87	94	7	98	yes	5.7E-04	4.8E-02
egl-27 + egr-1	97	99	83	93	73	62	yes	2.9E-01	5.7E-08

Table 3.4. Combinatorial RNA interference can identify known synthetic lethal interactions

Quantitative analysis of known synthetic lethal interactions ('Interaction Gene1 & Gene2'; see below for references) after combinatorial RNA interference (RNAi) in wild-type and *rrf-3* worms, respectively. Percentages of average wild-type brood size ('BS')

larvae, unhatched eggs and adult worms after combinatorial RNAi (performed in triplicates within independent screens) and RNAi against each gene individually (performed in duplicates within independent assays) in two separate experiments (see Figure 3.6. for an overview of the setup for combinatorial RNAi; see Materials and Methods for a detailed description). I normalized measurements for brood size and embryonic survival to wild-type measurements obtained after feeding worms on non-targeting dsRNA-expressing bacteria (Ahringer feeding library clone Y95B8A_84.g). If brood size or embryonic survival rates after RNAi against individual genes exceeded average wild-type measurements, I set values to 100% of wild-type values.

For statistical analysis, I compared the observed quantitative phenotypes resulting from simultaneously targeting both genes of a synthetic lethal pair with the calculated products of measurements for both genes individually. Therefore, I multiplied duplicate brood size and embryonic survival measurements, respectively, for both individual genes within two independent experiments in all possible pairwise combinations to generate sixteen values; these sixteen calculated products represent the predicted outcome if the double mutant phenotype was merely resulting from a simple additive effect of both single-gene RNAi phenotypes. The sixteen values for the expected quantitative phenotypes under a multiplicative model were compared to six measurements obtained after combinatorial RNAi in two independent experiments, using a Student's t-Test (two-tailed distribution, two-sample equal variance; see Materials and Methods for a detailed description of the statistical analysis). I considered SL interactions to be successfully recapitulated by combinatorial RNAi if p-values were below 5.0×10^{-2}.

Using the above criteria, I was able to detect all seven tested genetic interactions in *rrf-3* animals (Table 3.4., Figure 3.7.). However, in wild-type animals, only five of these interactions could be recapitulated (Table 3.4.). Not only did I fail to detect two out of seven interactions in wild-type worms, the five detected interactions were also weaker than in *rrf-3*, demonstrating that for effective combinatorial RNAi it is often essential to use RNAi-hypersensitive strains.

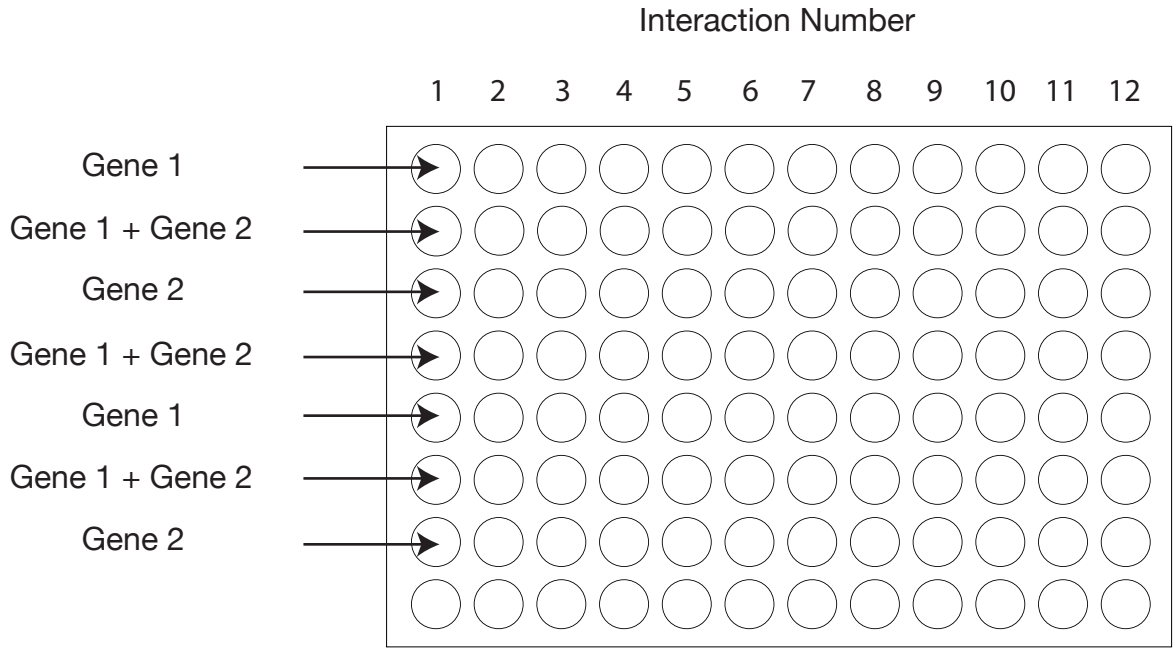

Figure 3.6. Overview of the setup for combinatorial RNA interference screens
When screening for genetic interactions, phenotypes resulting from simultaneously targeting two genes by combinatorial RNA interference (RNAi) were directly compared with the RNAi phenotypes of each gene alone. Genetic interaction screens are therefore set up such that worms were fed with bacteria expressing double-stranded RNA (dsRNA) against one gene ('Gene1'), with equal amounts of bacteria expressing dsRNAs against each of the two genes ('Gene1 + Gene2'), and bacteria expressing dsRNA against the second gene ('Gene2') in alternating rows. Using this setup, combinatorial RNAi was performed in triplicates and RNAi against each gene individually in duplicates within independent screens.

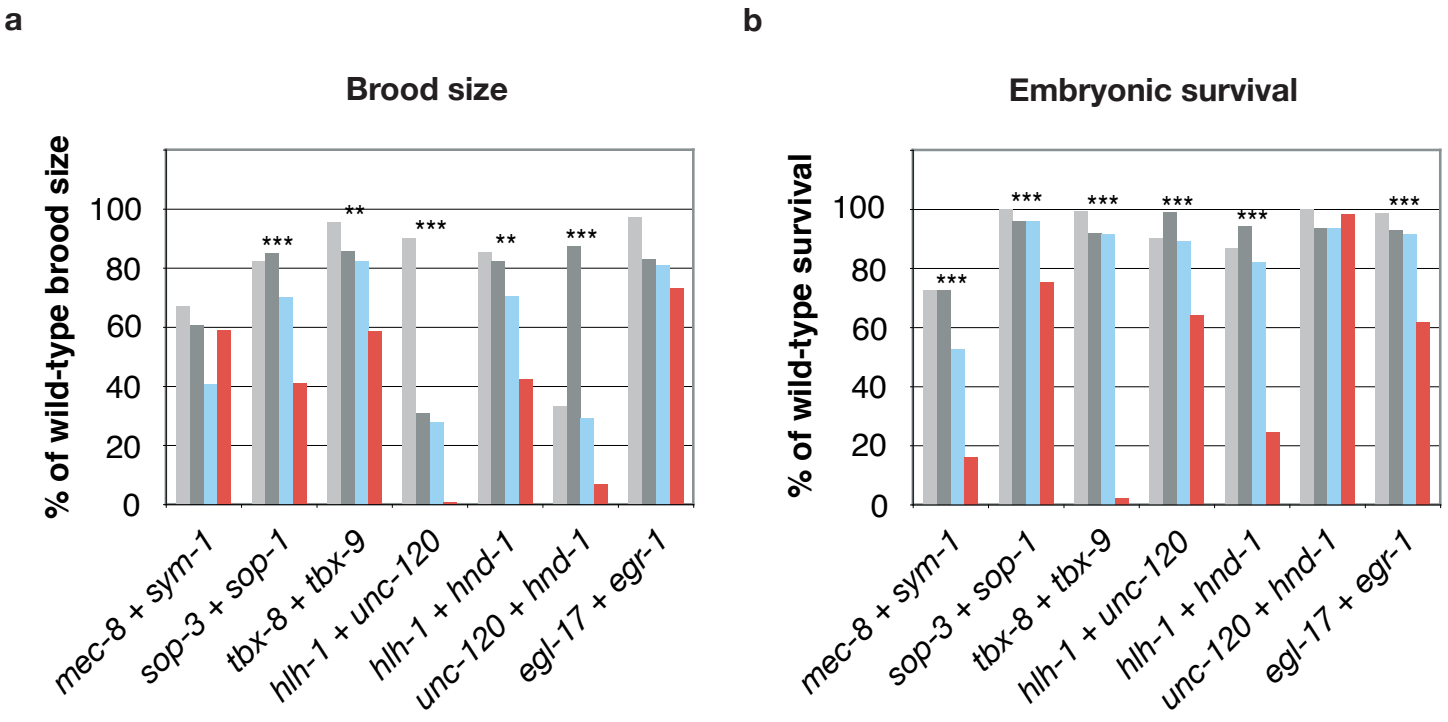

Figure 3.7. Combinatorial RNA interference can recapitulate known synthetic lethal interactions
To test whether combinatorial RNA interference (RNAi) could recapitulate seven synthetic lethal (SL) interactions that were identified from literature (see Table 3.4. for references), brood size (BS, a) and embryonic survival (ES, b) measurements following co-targeting of both genes of a SL pair (red bars) were compared with that following the targeting of each single gene alone (light- and dark-grey bars) and with the calculated product of the single gene brood sizes and embryonic survival measurements, respectively (blue bars). Values plotted represent the percentage of average wild-type brood size and embryonic survival rates, and are the arithmetic mean of two independent experiments performed in the RNAi-hypersensitive strain *rrf-3*. BS and ES measurements, respectively, resulting from combinatorial RNAi against both genes of a pair had to be significantly lower ($P < 5.0E-02$, Student's t-test) than the expected multiplicative values associated with BS and ES rates, respectively, after RNAi against each gene individually for a gene pair to be considered SL. ***, $P < 1.0E-03$; **, $P < 1.0E-02$.

Taken together, these results demonstrate that combinatorial RNAi by feeding using our newly developed HTP liquid-feeding platform is a robust and efficient method to simultaneously perturb the expression of any two genes in the genome of *C. elegans*. I was able to generate additive phenotypes and to detect the great majority of previously described synthetic post-embryonic phenotypes and synthetic lethal interactions. However, for effective combinatorial RNAi, it is often essential to use RNAi-hypersensitive strains. I thus decided to perform all of the following experiments in the RNAi-hypersensitive *rrf-3* background.

3.2.4. Effect of dilution on phenotype strength

When analysing the phenotypes produced through combinatorial RNAi, I and others (Gonczy *et al.*, 2000; Parrish *et al.*, 2000) observed that some of the single-gene phenotypes were qualitatively weaker when two genes were targeted together than when each gene was targeted alone. Since such dilution effects will affect both the false-negative rate in large-scale screens and the possible number of genes that can be co-targeted effectively, I wished to investigate the extent to which combining dsRNA-expressing bacteria leads to a reduced strength of RNAi phenotypes. To do this, I selected 282 genes from chromosome III that were found to have a non-viable (embryonic lethal or sterile) RNAi phenotype (Kamath *et al.*, 2003) (see Appendix Table 3.2.) and examined whether their phenotypes change as the targeting bacteria are diluted with increasing amounts of unrelated dsRNA-expressing bacteria (Figure 3.8.).

Using the HTP liquid-feeding assay, I compared RNAi phenotypes in the RNAi-hypersensitive strain *rrf-3* for each gene alone with RNAi phenotypes generated by diluting individual bacterial feeding cultures two-, three-, four-, five-, and ten-fold, respectively, with bacteria expressing a dsRNA that does not target an expressed portion of the *C. elegans* genome (Ahringer library clone Y95B8A_84.g), and bacteria expressing dsRNA against *lin-31*, respectively. For each gene with a non-viable RNAi phenotype in my experimental setting, I assessed the dilution level that first led to a drop in strength of phenotypes. I observed that the strength of RNAi phenotypes for many genes is indeed reduced when increasing proportions of bacteria expressing unrelated

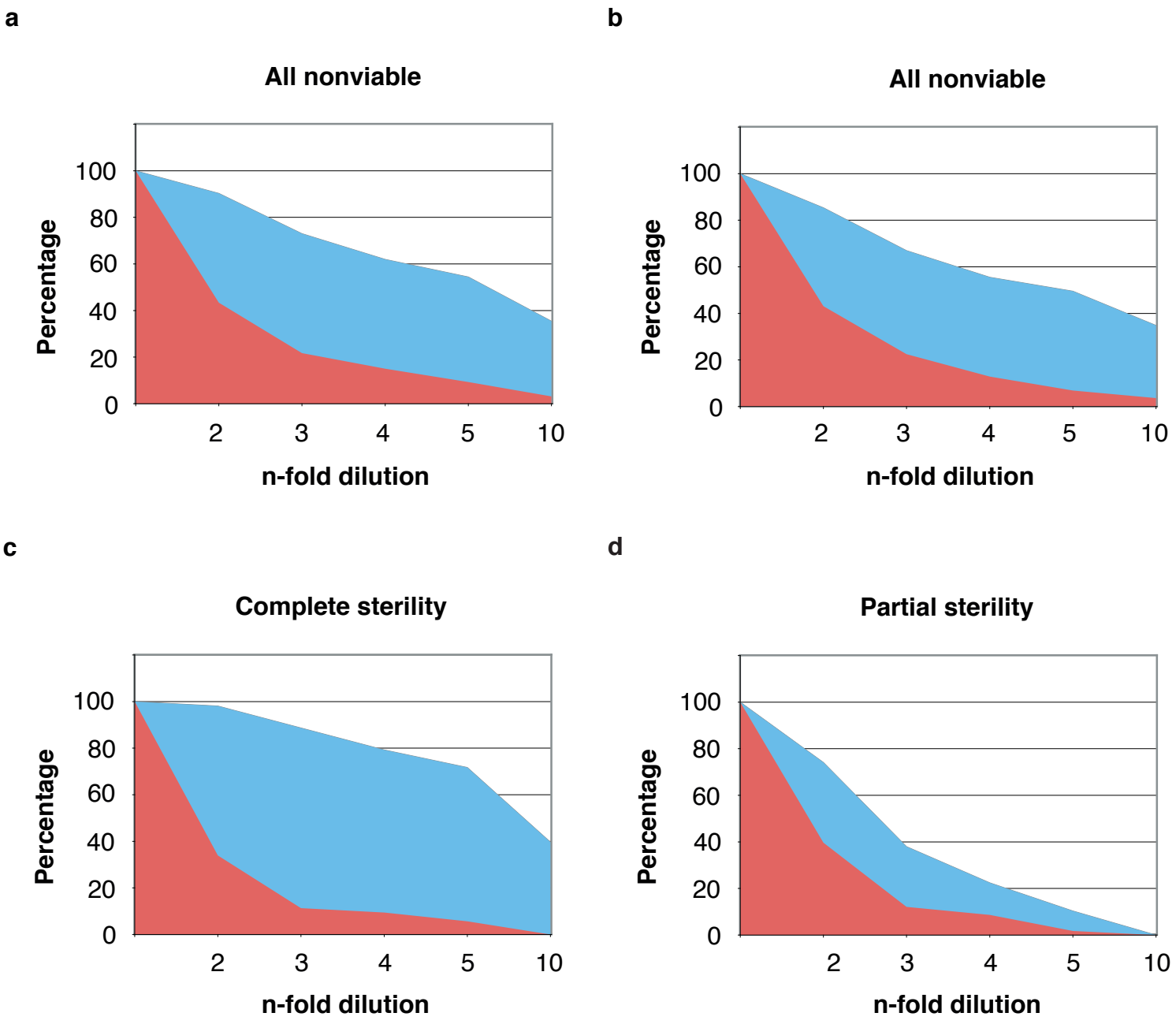

Figure 3.8. Effect of dilution on strength of RNA interference phenotypes
The RNA interference (RNAi) phenotype of each reported non-viable gene on chromosome III (Kamath *et al.*, 2003) was assessed following dilution with increasing amounts of bacteria expressing unrelated double-stranded RNAs (dsRNAs). The percentage of genes with phenotypes that are either identical to that observed when targeted alone (red) or weaker than when targeted alone (blue) is shown for each dilution. This was examined for three phenotypic categories: all non-viable phenotypes (dilution with bacteria expressing a non-targeting dsRNA (Ahringer feeding library clone Y95B8A_84.g; a) and dilution with bacteria expressing dsRNA against *lin-31*; b), complete sterility (no progeny; dilution with non-targeting dsRNA-expressing bacteria; c), and partial sterility (some progeny; dilution with non-targeting dsRNA-expressing bacteria; d). Data shown are representative of two independent experiments performed in the RNAi-hypersensitive *rrf-3* background.

dsRNAs are added. I found essentially identical results when diluting with non-targeting dsRNA-expressing bacteria (Ahringer library clone Y95B8A_84.g; Figure 3.8.a), as when adding increasing amounts of a dsRNA-expressing bacterial strain targeting *lin-31* (Figure 3.8.b), demonstrating that the observed effect is not specific to the diluting dsRNA-expressing strain. I will discuss the observed changes in phenotypic strength in more detail below, focusing on results obtained with increasing dilution of bacteria expressing a non-targeting dsRNA (Ahringer library clone Y95B8A_84.g).

In total, I was able to detect phenotypes for ~90% of genes with non-viable RNAi phenotypes when the targeting strains were diluted with equal amounts of a bacterial strain expressing an unrelated dsRNA. This detection rate dropped further to ~70% at three-fold and to ~60% at four-fold dilution.

I next asked whether the effect of dilution on the observed phenotype is related to phenotypic strength. To this end, I determined the dilution behaviour for genes that have different strengths of brood size defects when targeted alone (Figure 3.8.c and 3.8.d). I found that genes with weak RNAi phenotypes were indeed more likely to appear wild-type following dilution — and thus to be missed in screens — than genes with strong, highly penetrant phenotypes. For example, I could still detect phenotypes for ~80% of genes, that normally have a completely sterile phenotype, at a four-fold dilution (Figure 3.8.c); however, only ~20% of genes conferring partial sterility (i.e. a reduction in brood size) have a detectable phenotype at this dilution (Figure 3.8.d). While this indicates that genes with weaker phenotypes are more likely to appear wild-type when targeted in combination with other genes, I conclude that on average ~90% of genes with a detectable RNAi phenotype still have sufficient knockdown when diluted with equal amounts of a second dsRNA-expressing bacterial strain.

Overall, these experiments allowed me to estimate the false-negative rates induced by dilution effects in combinatorial RNAi (Figure 3.9.; see Materials and Methods for calculation). Assuming each gene behaves independently, I expect that ~80% of bigenic interactions yielding visible RNAi phenotypes will be detectable by combinatorial RNAi using the HTP liquid-feeding assay.

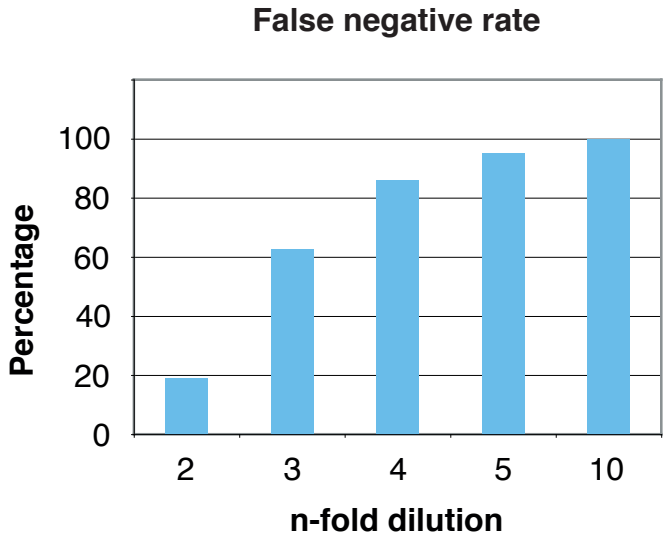

Figure 3.9. False-negative rate of combinatorial RNA interference
The false negative rate (in percent) of combinatorial RNA interference (RNAi) at a given dilution was calculated by assessing the fraction of chromosome III genes with nonviable RNAi phenotypes that resulted in a weaker phenotype when increasing amounts of non-targeting double-stranded RNA- (dsRNA-) expressing bacteria were added to targeting dsRNA-expressing bacteria. Data shown are representative of two independent experiments in the RNAi-hypersensitive strain *rrf-3*.

In summary, I have systematically analysed the effects of diluting bacteria expressing dsRNAs against genes with known non-viable RNAi phenotypes with increasing amounts of unrelated dsRNA-expressing bacteria. I found that combining equal amounts of two dsRNA-expressing bacterial strains frequently results in a reduced strength of phenotypes. The implication of this dilution effect for combinatorial RNAi is that as one increases the number of genes being simultaneously targeted, the efficiency of the knockdown of each individual gene decreases. Thus, one cannot increase the number of genes being co-targeted indefinitely. However, the great majority of genes with sterile or embryonic lethal RNAi phenotype still showed non-viable phenotypes at two-fold dilution. This dilution is critical for the study of bigenic interactions using combinatorial RNAi. Thus, together with the generation of additive phenotypes within the same animal and the detection rate of known synthetic genetic interactions, these findings suggest that combinatorial RNAi using a HTP liquid-feeding assay is a powerful tool for systematically studying the effect of targeting any pairwise combination of genes in the genome of *C. elegans*. I conclude that this approach should allow researchers to explore genetic interactions in the nematode *C. elegans* in a far more systematic manner than has been possible in the past.

3.3. Conclusion

In this chapter, I have shown the development of an experimental platform for using RNAi by bacterial feeding to analyse the loss-of-function phenotypes of single genes on a large scale. By delivering dsRNA-expressing bacteria in liquid cultures in 96-well format, this methodology allows RNAi screens to be performed at considerably higher throughput than has been possible in the past. Notably, this HTP liquid-feeding assay allows the screening of a population of worms in each well, thereby averaging the animal-to-animal variability of RNAi phenotypes that is observed when using single-animal plate-feeding protocols. By using this assay, I was able to identify approximately 80% of previously identified RNAi phenotypes with 90% reproducibility.

I then established protocols to adapt these methods, which are very robust and efficient for analyzing the RNAi phenotypes of single genes, to simultaneously targeting

two genes by mixing two dsRNA-expressing bacterial strains (‘combinatorial RNAi’). Using this methodology, I was able to generate loss-of-function phenotypes for two genes in the same animal and to identify the great majority of a test set of previously known synthetic lethal and synthetic post-embryonic genetic interactions.

When investigating the extent to which combining dsRNA-expressing bacteria can lead to a reduced strength of phenotype, I was able to detect phenotypes for approximately 90% of genes with a non-viable RNAi phenotype at two-fold dilution. This is the critical dilution that is needed for detecting bigenic interactions by using combinatorial RNAi. Assuming that each gene behaves independently in combinatorial RNAi, I expect that approximately 80% of bigenic interactions yielding visible RNAi phenotypes will be detectable by using this approach.

Taken together, I consider combinatorial RNAi by bacterial feeding a powerful tool for examining interactions between any pair of genes in the genome of *C. elegans* on a large scale.

Having established and validated combinatorial RNAi as a robust high-throughput method for simultaneously targeting any pairwise combination of genes by using bacterial feeding in liquid culture, I wished to use this approach to begin to investigate functional redundancy in the genome of *C. elegans*.