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Tuning degradation to achieve specific and efficient protein depletion

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Corresponding Author:	David Beggs UNITED KINGDOM
Corresponding Author's Institution:	
Corresponding Author E-Mail:	David.Barrass@ed.ac.uk
Order of Authors:	David Barrass
	Gonzalo Mendoza-Ochoa
	Isabella Maudlin
	Emanuela Sani
	Jean D Beggs
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TITLE:

Tuning Degradation to Achieve Specific and Efficient Protein Depletion

AUTHORS AND AFFILIATIONS

J. David Barrass[1], Gonzalo I. Mendoza-Ochoa[1,2], Isabella E. Maudlin[1], Emanuela Sani[1], Jean D. Beggs[1]

[1]Wellcome Centre for Cell Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

[2]Present address: Department of Plant Sciences, University of Cambridge, Cambridge, UK

Corresponding Author

David Barrass

david.barrass@ed.ac.uk

KEYWORDS:

AID, auxin, β-estradiol, degron, protein depletion, regulated gene expression, *Saccharomyces cerevisiae*

SUMMARY

Here, we present a protocol to effectively and specifically deplete a protein of interest in the yeast *Saccharomyces cerevisiae* using the β-est AID system.

ABSTRACT

The plant auxin binding receptor, TIR1, recognizes proteins containing a specific auxin-inducible degron (AID) motif in the presence of auxin, targeting them for degradation. This system is exploited in many non-plant eukaryotes, such that a target protein, tagged with the AID motif, is degraded upon auxin addition. The level of TIR1 expression is critical; excessive expression leads to degradation of the AID-tagged protein even in the absence of auxin, whereas low expression leads to slow depletion. A β-estradiol-inducible AID system was created, with expression of TIR1 under the control of a β-estradiol inducible promoter. The level of TIR1 is tunable by changing the time of incubation with β-estradiol before auxin addition. This protocol describes how to rapidly deplete a target protein using the AID system. The appropriate β-estradiol incubation time depends on the abundance of the target protein. Therefore, efficient depletion depends on optimal timing that also minimizes auxin-independent depletion.

INTRODUCTION

Conditional mutations, such as temperature-sensitive mutants, are a powerful tool for the study of essential proteins, allowing cell growth under the permissive condition but causing loss of function under non-permissive conditions. However, cell metabolism can be seriously perturbed by the change in growth conditions required to induce the defect and may also create off-target effects. Several methods have been developed, in which the protein of interest is conditionally sequestered[1] or its expression is controlled[2,3] by addition of a small molecule. This protocol uses auxin and the auxin-inducible degron (AID) system to efficiently deplete a target protein.

The AID system has its origin in plants, where an auxin (in this protocol indole-3-acetic acid (IAA) is used), stimulates interaction of the Aux/IAA protein with TIR1, a member of the SCF U3 ubiquitin ligase complex[4]. SCF complex interaction causes polyubiquitination of Aux/IAA family proteins, which results in their degradation by the proteasome[5,6].

This system was previously adapted for use in the yeast *Saccharomyces cerevisiae*[7,8] by expressing the TIR1 protein from *Oriza sativa* (osTIR) in yeast cells, where it is able to interact with the endogenous yeast SCF complex. The protein of interest was tagged with a motif from the Aux/IAA protein IAA17 to target it for degradation. Functional truncations of IAA17 were developed later, such as AID* [8-10], containing the 43 amino acid auxin-sensitive motif from *Arabidopsis thaliana IAA17*, along with an epitope tag to enable detection .

The system initially adapted for use in budding yeast[7,8] expressed the osTIR1 protein from a yeast GAL promoter. Expression requires shifting to growth medium with galactose as the sole carbon source, which, unfortunately, results in a diauxic shift with wide-ranging changes to cell metabolism[11]. On the other hand, it has been reported that constitutive expression of TIR1 can lead to degradation of the target protein in the absence of auxin/IAA[12] if the expression level is high, whereas low TIR1 expression causes inefficient depletion. An improved AID system named β-est AID was developed in which the osTIR is under the control of an inducible promoter that is tunable to suit the target protein, with minimal effect on cell metabolism. To achieve this, an artificial transcription factor (ATF) was constructed in which the *VP16* viral transcription activator is fused to an oestrogen receptor and a four Zn fingers DNA binding domain (DBD). When β-estradiol (an oestrogen) is present, the ATF can enter the nucleus and induce osTIR transcription by binding to its promoter (Z4EVpr)[13,12] .

osTIR expression is usually detectable about 20 min after addition of β-estradiol[12]. However, the optimal duration of osTIR expression to achieve efficient depletion of the tagged protein with auxin, while avoiding depletion before auxin addition, needs to be empirically determined for each target protein. An approximate time for this pre-incubation can be estimated from abundance values in the Saccharomyces Genome Database (SGD https://www.yeastgenome.org/). As can be seen in **Figure 1**, the abundant protein, Dcp1 (2880 to 4189 molecules/cell), requires 40 min of pre-incubation with β-estradiol, with no auxin-independent depletion observed. The much less abundant protein, Prp2 (172 to 211 molecules/cell), is strongly depleted after only 20 min of pre-incubation. It is advisable to test two additional pre-incubation times, 10 to 20 min before or after this initial estimated time (20 min is the minimum time that is recommended). The optimum pre-incubation time is the time at which target protein has not depleted before adding auxin and once auxin is added the depletion is acceptable or protein levels approach the minimum possible. So, from **Figure 1b**, for Prp22 with 30 min of pre-incubation, the levels have not declined much 10 min after auxin addition. Comparing this with 40 min of pre-incubation and 15 min with IAA, where there is little additional depletion, there is no benefit in incubating with auxin longer than 10 min or pre-incubating for longer than 30 min, particularly as there is evidence of non-auxin dependent depletion at 40 min. For Dcp1 with 40 min of pre-incubation (the last point at which the protein level is approximately 100% before auxin addition), 15 to 20 min of depletion with auxin is acceptable. It is recommended to keep the depletion time as short as possible to reduce secondary effects on cell metabolism[14].

This article demonstrates how to use the β-est AID system by optimizing the timing of β-estradiol incubation for osTIR expression to achieve rapid target protein depletion upon IAA addition without depletion before adding auxin.

PROTOCOL:

NOTE: See **Figure 2** for a graphical summary.

1. Strain preparation

1.1. Using a *ura3*-strain, introduce the β-est AID system (i.e., genes encoding the β-estradiol responsive transcription factor (ATF) and the osTIR) and AID* tag the target protein (see **Figure 3** and **Table 1** for a summary of the procedure).

1.1.1. Transform[15] either pZTRK (G418 resistance marker) or pZTRL (*LEU2* marker) plasmid (available from the Yeast Genetic Resource Centre) into the *ura3*- yeast strain or use the plasmid as a template to produce the PCR product for genomic integration.

1.1.2. PCR amplify the ATF (marked Z4EVATF on the plasmid map) and osTIR using a high fidelity polymerase from either of the plasmids pZTRK or pZTRL. Use primers with 50 to 60 base 3' extensions with homology to the genomic region, to direct integration by homologous recombination[16]. For genomic integration of the two components either separately or together, see **Table 1** for primers and conditions.

NOTE: The strain pZ4EV-NTR1 has the components already integrated in the genome (available from the Yeast Genetic Resource Centre, Japan).

1.1.3. Ensure that the target protein is AID* tagged using the Longtine procedure[17] (see **Figure 3b** and **Table 1**).

1.1.4. Perform a growth analysis on the strain without β-estradiol and IAA present to determine if the AID* tag affects growth and to predict growth rate for use at step 1.5.

2. General procedure for depletion

2.1. Calculate how much culture is required for all samples to be collected; for example, 10 mL of culture at OD_{600} of 0.8 is sufficient for protein, RNA and DNA extraction for a single sample, so for 6 samples, at least 60 mL of culture is needed.

2.2. From an overnight culture, set up sufficient new culture at OD_{600} 0.1 to 0.2 and leave to grow at 30 °C. A rich medium such as YPDA is recommended, although other growth conditions can be used:

Yeast Extract	10 g
Peptone	20 g
Glucose	20 g
Adenine sulphate	40 mg
H_2O to	1 L

NOTE: Autoclave or filter sterilize; filter sterilization is preferred as peptide/sugar complexes produced by autoclaving precipitate in the methanol used in sample collection.

2.3. Prepare to receive the samples.

2.3.1. Put 30 to 50% of the intended sample volume of methanol into a tube. For example, if a 10 mL sample is to be taken, put 5 mL of methanol into a 15 mL falcon tube and close the tube tightly. Once closed, label the tube and put on dry ice or at -80 °C to chill.

CAUTION: Dispense the methanol in a fume hood.

2.3.2. Label 1.5 mL tubes for long term storage of the samples and place in ice to cool.

2.3.3. Cool enough H_2O (at least 1 mL per sample) on ice.

2.4. Anticipate the culture's growth. The target OD for collecting the samples is approximately 0.7 to 0.8, but the pre-incubation step (the incubation with β-estradiol to induce the osTIR), needs to be started earlier so that the culture will reach approximately the right OD by the time the samples are collected.

NOTE: It is advisable to perform a growth curve in the conditions to be used in the experiment so that this starting OD can be estimated.

2.5. Once the target OD for the start of the pre-incubation has been reached, take a sample (usually 10 mL), into the pre-prepared tube containing cold methanol. Invert briefly to mix and place back in dry ice.

NOTE: The sample can be moved to water ice after about 5 min, if convenient to do so.

2.6. Immediately add the β-estradiol, 1 µL/mL of culture (final concentration of 10 µM); have the β-estradiol pre-measured in a pipette ready for use in order to reduce the time taken between collecting the sample and adding the β-estradiol. Rapidly mix by swirling vigorously.

2.7. Continue to grow the culture as before (step 2.2), incubate (this is the "pre-incubation" step) with β-estradiol for the optimal time (for determination of the optimal pre-incubation time see **Figure 1**).

2.8. Prepare to add IAA (auxin). Take up the volume of IAA needed for step 2.10 (i.e., 0.5 µL of IAA per mL of culture). This makes step 2.20 faster.

2.9. Collect a sample as step 2.5.

2.10. Immediately add IAA 0.5 µL/mL of culture to a final concentration of 750 µM as prepared in step 2.8. Rapidly mix by swirling vigorously.

2.11. Collect samples, as step 2.5, according to your experimental design. Either a single sample, at a time when it is expected that the protein will be reliably depleted, or multiple samples in a time course of depletion. For example, 5 min intervals are convenient for timing and provide a range of protein levels. The optimization strategy, as shown in **Figure 1**, will give an indication of suitable times.

2.12. Process the samples.

2.12.1. Place the samples on ice, if not done already. Ensure that none of the samples has frozen; if they have, gently warm in the hand, inverting constantly so the temperature does not rise locally.

NOTE: This is best done in the hand as the sample's temperature can be assessed, it should always feel cold. Place on ice. This is not a pause point - once all the samples are fluid, proceed to the next step.

2.12.2. Once all samples have been collected and are no longer frozen, spin at 3,500 x *g* for 2 min (at 4 °C if possible).

2.12.3. Pour off the methanol/medium mix and place back on ice; do not worry if not all the liquid has been removed.

2.12.4. Resuspend the cell pellet in 1 mL of ice cold H_2O (from 2.3.3) and transfer to a labelled 1.5 mL tube (prepared in step 2.3.2) on ice.

2.12.5. Spin briefly (e.g., 10 s total time) at >15,000 x *g* to re-pellet the cells, place back on ice and remove the liquid.

2.12.6. Remove the H_2O by aspiration. The cell pellets can be stored at -20 °C, or -80 °C for long term storage.

2.13. Check the level to which the protein has been depleted by Western blot analysis[18].

NOTE: Sufficient protein[19] and/or nucleic acid can be extracted from a single cell pellet for most purposes, although rare RNA species might require more sample volume.

REPRESENTATIVE RESULTS

Representative examples of depletion are displayed in **Figure 1**. The three experiments presented in this figure were optimization experiments for depletion of the proteins Prp2, Prp22 and Dcp1. The low abundance, spliceosomal Prp2 and Prp22 proteins both depleted to less than 20% after 40 min pre-incubation with β-estradiol followed by 15 min with auxin. In comparison, the more abundant Dcp1 was only depleted to approximately 30% with the same treatment, but 60 min of pre-incubation resulted in depletion to 13% with the same auxin treatment, at the cost of depletion before the auxin is added. It is possible that 50 min of pre-incubation with β-estradiol and 15 min with auxin would have achieved similar results at a shorter time point and so would have been more optimal.

FIGURE LEGENDS

Figure 1. Depletion rate can be tuned by modulating the duration of β-estradiol pre-incubation. Western blot[18] of target proteins: (**a** and **b**) Prp22-AID*-6FLAG, (**c** and **d**) Prp2-AID*-6FLAG, and (**e** and **f**) Dcp1-AID*-6HA, from cultures pre-incubated with β-estradiol (β-est) for 20, 30, 40, or 60 min prior to auxin addition[12]. Equal amounts of total protein were loaded in each lane. Pgk1 is detected as a visual loading control, except for panel **e**, where Pgk1 and Dcp1 co-migrate. Quantification of protein bands in panels **a**, **c** and **e** are shown in panels **b**, **d**, and **f,** respectively. As a measure of depletion rate, the slope (m) was calculated for the linear section (from 100% to 30% of initial values) of each curve. The optimal pre-incubation time is the time at which the protein levels are still close to the un-induced levels (100%) and the subsequent rate of depletion is fast. For Dcp1 (**f**), 60 min of pre-incubation is too long, as the protein has begun to degrade in the absence of auxin, whereas 20 min is too short, as the protein does not appreciably deplete in this time course. After 40 min pre-incubation, 15 min with auxin can be used as the protein is approximately 70% depleted and, although 20 min would result in further depletion, it could also result in secondary effects. Error bars represent standard deviation of two biological replicates. For each experiment, one representative blot is shown. This figure is derived from previous publication[9].

Figure 2. Graphical summary. Add β-estradiol to sufficient culture growing in rich medium and at the required temperature in order to start pre-incubation. Continue growth for the required pre-incubation time before adding IAA (auxin) to start depletion. The pre-incubation and depletion times depend on the protein to be depleted, but pre-incubation is often in the range of 20-60 min and the depletion time is typically in the order of 10 to 20 min. 10 mL samples should be taken at the start and end of pre-incubation and during the depletion. These samples are rapidly fixed in cold methanol before pelleting and storage.

Figure 3. Strain generation for the B-est system. (**a**) To generate a yeast strain with the AID* system, either the pZTRL (*LEU2*) or the pZTRK (kanamycin (G418) resistance), plasmid should be introduced into the strain or, alternatively, the ATF and osTIR can be inserted into the genome by homologous recombination of a fragment generated by PCR from 3'end primers (see **Figure 3b** and **Table 1**). (**b**) C-terminal tagging of the target protein is achieved by PCR amplification of the appropriate region of the plasmid pURA3-AID*-6FLAG (pURA3_AID*-6HA differs only in the tag and can be treated in exactly the same way), using Longtine primers S3-F and S2-R with 3' extensions homologous to the 3' end of the target protein. The forward primer extension should include the last amino acid codon in frame with the start of the AID* tag and must not include the stop codon. The reverse primer extension should be to a region downstream of the coding region. Once inserted into the genome, cells that have lost the *URA3* marker (by homologous recombination between the identical regions found at both ends of the marker) can be selected by growth with 5-FOA, that counter-selects *URA3* cells.

Table 1. Primer sequences, PCR mix and PCR conditions.

DISCUSSION

A well optimized protocol can produce rapid and efficient depletion of the target protein. Determining the approximate pre-incubation time with β-estradiol is important, as this increases reproducibility of the depletion, but small variations in pre-incubation time can be

tolerated. On the other hand, care must be taken with timing after auxin addition, as the protein level declines very rapidly.

An advantage of this approach is that tuned depletion can be achieved by varying combinations of pre-incubation time with β-estradiol and IAA incubation time. For example, if desired, the target protein can be more slowly depleted by reducing the pre-incubation time.

The β-est AID system offers certain advantages over systems where OsTIR is constitutively expressed. For example, if the target protein is essential for viability, regulated expression of osTIR can avoid premature depletion of the target protein. Moreover, expression of osTIR can be tuned to suit the abundance of the target protein and its susceptibility to degradation, and the depletion can be either fast or slow. The two small molecule effectors, β-estradiol and auxin, do not perturb the yeast metabolism under the conditions used here, unlike rapamycin, used in the anchor-away system[1].

It should be noted that tagging some proteins disrupts their function, which is a problem with any targeted depletion system. In this case, an N-terminal tag may work when a C-terminal tag does not. Also, not all proteins will be depleted efficiently; for example, the AID-tag on the target protein may be inaccessible to the osTIR protein. Therefore, after AID-tagging, each target protein should be tested for any effect of the tag on growth, and to determine whether depletion is effective, before the timings of β-estradiol pre-incubation and auxin treatment are optimized.

This AID* system is very simple and is compatible with any subsequent experimental procedure that does not involve further growth, such as protein, DNA or RNA analysis or microscopy. In addition, the system works well when combined with thiolabelling to purify nascent RNA[20].

This system provides a rapid, specific, and reproducible means of depleting a protein without otherwise affecting the metabolism of the yeast cell.

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DISCLOSURES

The authors have nothing to disclose.

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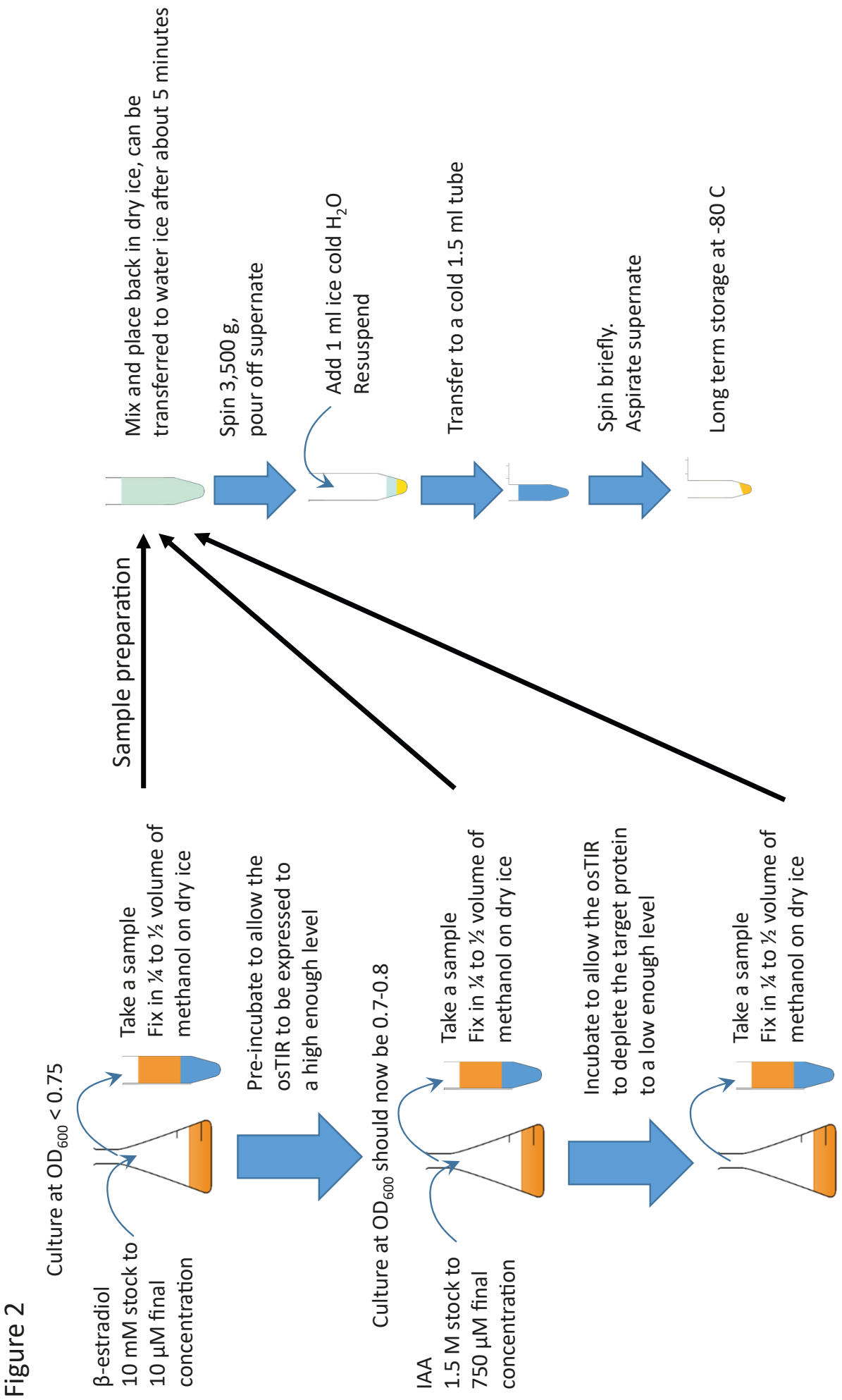

Figure 1

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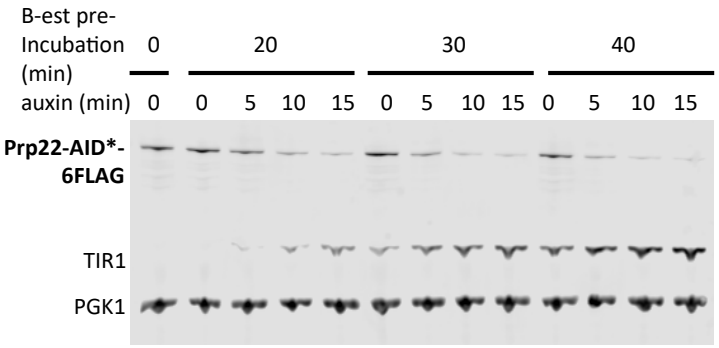
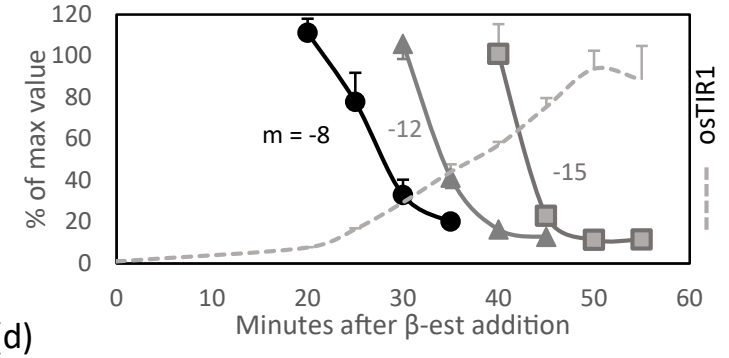
(a)

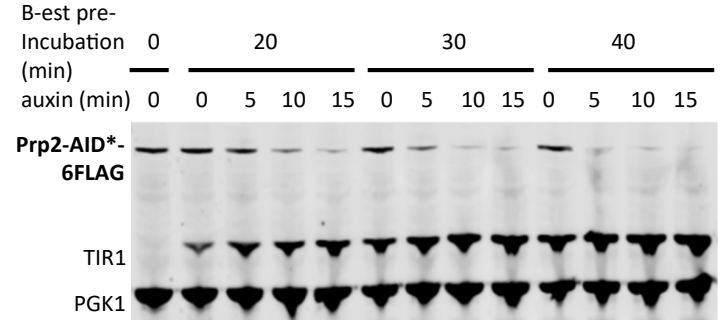


(b)

Prp22-AID*-6FLAG levels when pre-incubating with β-est for 20 (●) 30 (▲) or 40 (■) min prior to auxin addition

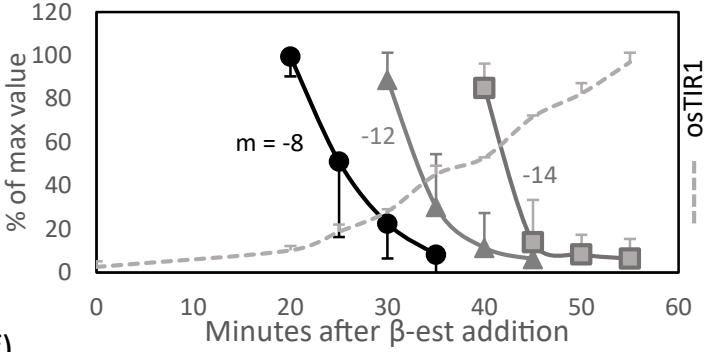


(c)

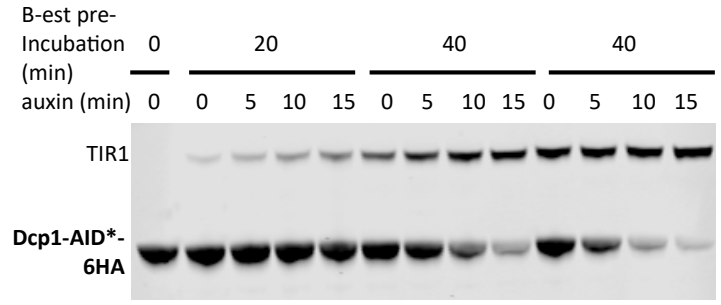


(d)

Prp2-AID*-6FLAG levels when pre-incubating with β-est for 20 (●) 30 (▲) or 40 (■) min prior to auxin addition



(e)



(f)

Dcp1-AID*-6HA levels when pre-incubating with β-est for 20 (●) 40 (■) or 60 (◇) min prior to auxin addition

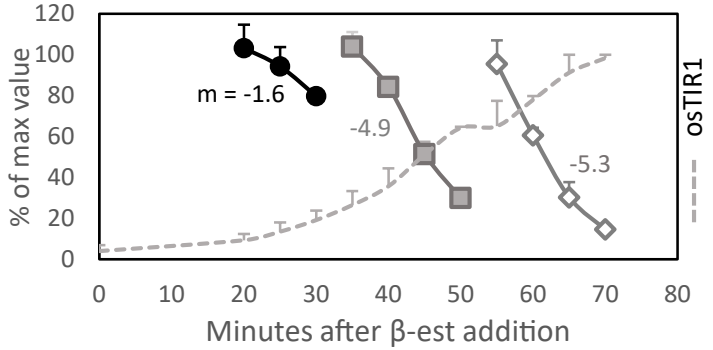
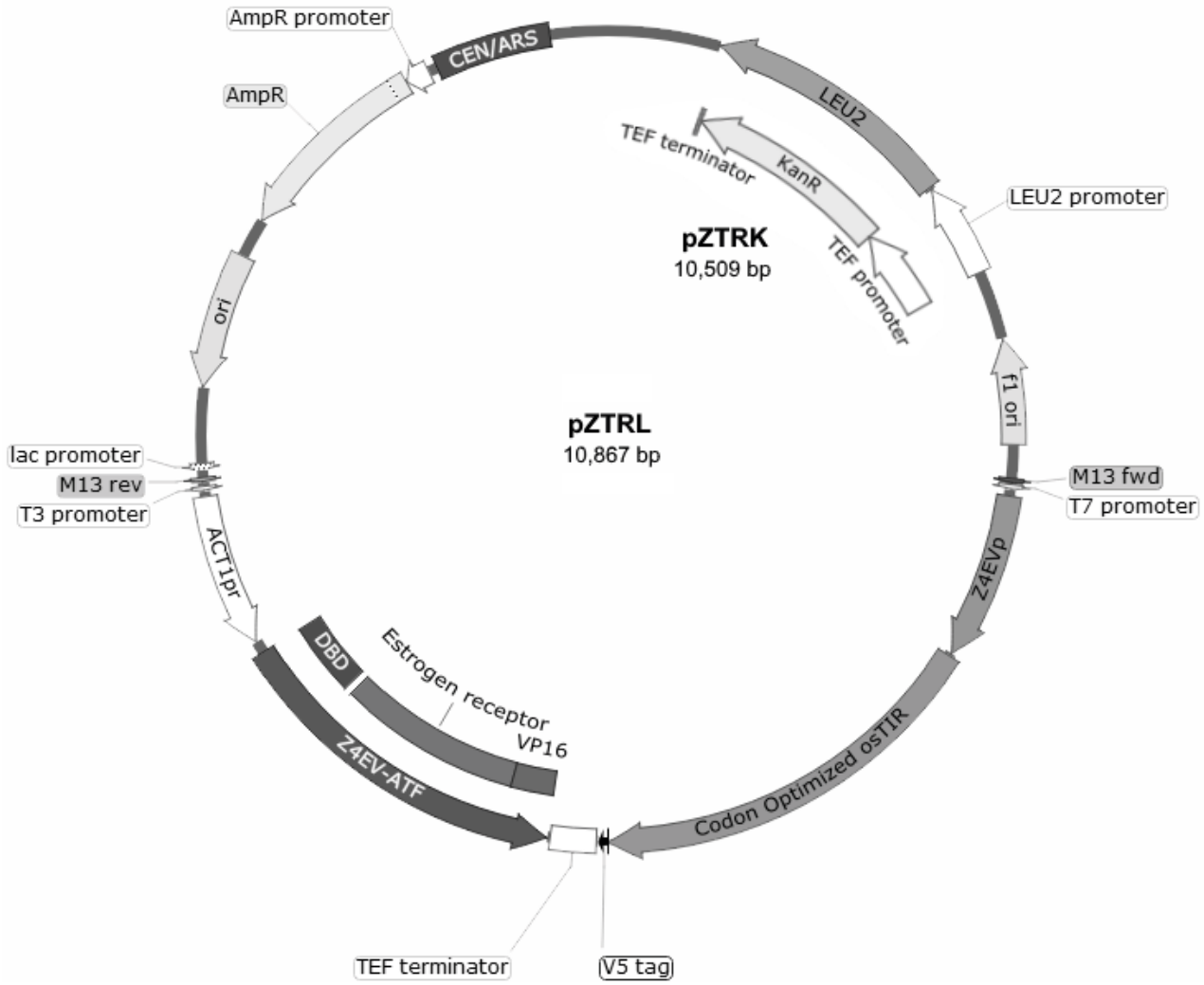


Figure 3

(a)



(b)

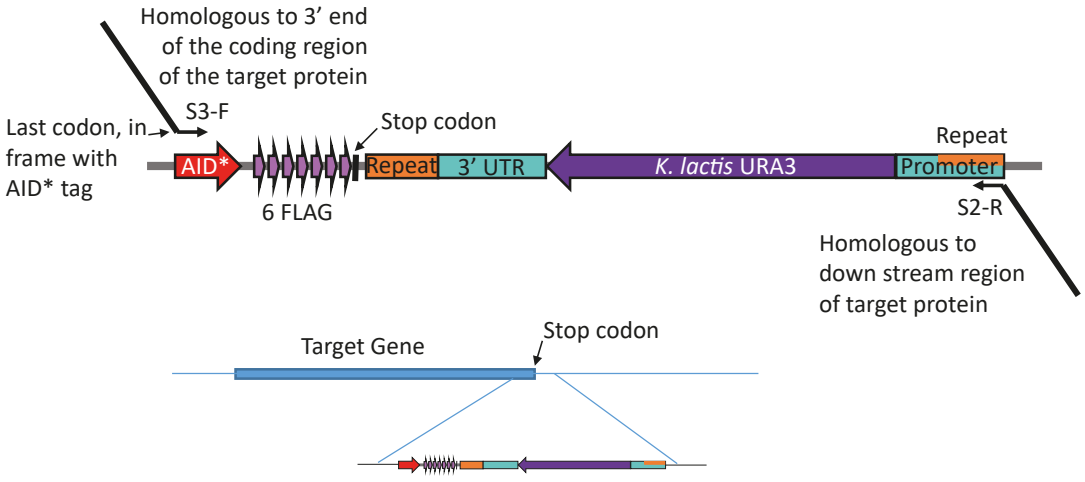


Table 1

a. Primer Sequences

Target	Location	Primer	Name	Sequence	Tm (°C)
pZTRL	516	F	pZTRL_F	<-region of homology->GCGACAGCATCACCGACTTCG	61.23
	7897	R	pZTR_R	CGCCGCCTCTACCTTGCAGA<-region of homology (RC)->	61.30
pZTRK	9154	F	pZTRK_F	<-region of homology->ACGTTGAGCCATTAGTATCAATTTGCTTACC	59.40
	5897	R	pZTR_R	CGCCGCCTCTACCTTGCAGA<-region of homology (RC)->	61.30
pURA3-AID*-6FLAG		F	S3-F	<-region of homology->CGTACGCTGCAGGTCGAC	59.21
or pURA3_AID*-6HA		R	S2-R	ATCGATGAATTCGAGCTCG<-region of homology (RC)->	52.76

pZRTL/K is to amplify the β-est AID system

pURA3-AID*-6FLAG/6HA to amplify the AID* and epitope tag to tag the target protein (Lontine procedure)

<-region of homology->	Region homologous to the flanking regions where the system is to be inserted. The longer this region is the more likely the modification is to be successful; 50 - 100 bases is recommended.
<-region of homology (RC)->	Region homologous to the flanking regions where the system is to be inserted, remember to use the reverse complement. As above, the longer this region is the better.
Tm (°C)	Tm using the %GC method with 50 mM NaCl

b. PCR Mix

Component	Volume (µl)
Template	<10
NEB Phusion HF Buffer (5 X)*	100
Forward Primer 100 µM	2.5
Reverse Primer 100 µM	2.5
dNTPs 10 mM each	10
H_2O	to 500

* The NEB Phusion GC Buffer (5 X) can also be used but is not preferred

Make this mix, split into 10 tubes of 50 µl mix each and perform the PCR as Table 1 c.

Check the PCR has worked by running on an agarose gel

Combine all successful reactions into one tube and ethanol precipitate

Transform the yeast with all the material produced by the PCR

c. PCR Conditions

Step		Temp (°C)	Time
Initial Denaturation		98	30 s
25-35 Cycles	Denature	98	10 s
	Anneal	45 - 60	20 s
	Extension	72	30 s/kb
Final Extension		72	10 min
Hold		8	

Anneal at 45°C for the Lontine primer set (S3-F and S2-R) and 60 °C for the pZTRL/K primers

Extend for 3 minutes for the Lontine PCR and 3 minutes for pZTRL/K

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adenine sulphate	Formedium	DOC0230	
Agar	Formedium	AGA03	
B-estradiol	Sigma Aldrich	E2758-1G	10mM solution in ethanol. Store at -20 ^{o}C
DMSO	Alfa Aesar	42780	DMSO should be solid at 4 ^{o}C
Glucose	Fisher Scientific	G/0500/60	
IAA 1H-Indole-3-acetic acid	Across Orgainics	122150100	Auxin analogue. 1.5 M in DMSO. The solution will be a russet colour and darken as time goes on; a deep red solution should be discarded and a new one made. Store at -20 ^{o}C.
Methanol	Fisher Scientific	M/4000/PC17	CAUTION Toxic and flammable
Phusion High-Fidelity DNA Polymerase	NEB	M0530	
Peptone	Formedium	PEP03	
SCSM single drop-out –ura	Formedium	DSCS101	
Yeast Extract	Formedium	YEA03	
Yeast nitrogen base without amino acids with amonium sulphate	Formedium	CYN0410	

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Title of Article:	Tuning degradation to achieve specific and efficient target protein depletion
Author(s):	J. David Barrass, Gonzalo I. Mendoza - Ochoa, Isabella E. Maudlin, Emanuela Sani, Jean D. Beggs

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CORRESPONDING AUTHOR

Name:	David Barrass
Department:	WTCCB
Institution:	University of Edinburgh
Title:	Mr
Signature:	David Barrass
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The manuscript has been modified and the updated manuscript, **59874_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. Step 1.1.1: Please ensure that all text is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.).
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10. 2.7: What’s the temperature for incubation?
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16. 3.12: Please ensure that all text is written in the imperative tense as if telling someone
We would recommend rewrite the steps as:
1.1 Use *ura3*- as the parental strain. Into this strain, introduce the β-est AID system (i.e. the β-estradiol responsive transcription factor (ATF) factor & the osTIR ORF).
1.1.1: Obtain a plasmid, either pZTRK (G418 resistance marker) or pZTRL (LEU2 marker) available from the Yeast Genetic Resource Centre. Transform the plasmid into a yeast strain or use as a template for genomic integration.
1.1.2: PCR amplify the ATF (marked Z4EVATF on the plasmid map) and osTIR using a high fidelity polymerase from either of the plasmids pZTRK or pZTRL. Use primers with 50 to 60 base 3’ extensions with homologies to the genomic region the insertion is to be placed in by homologous recombination. Integrate the two components genomically separately or together, see Table 1 for primers and conditions.
1.1.3 could be written as a note.

Steps 1.2.1, 1.23 and 1.2.4: We could not film generic steps. Please use a specific protein as an example.

Steps 2.2, 2.7 and 3.3: Please provide the conditions for your specific experiment. However, a note could be added, indicating that conditions depend on different experiment.

Authors Response
These sections have been completely re-written, and I feel that the new version is much more in-line with what is required for a Jove paper now. Thank you for your advice

17. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

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18. Figure 2: Please add a short description of the figure in Figure Legend.

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