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🌐 The ARF-AID system: Methods that preserve endogenous protein levels and facilitate rapidly inducible protein degradation

In 1 collection

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Abstract

The ARF-AID (Auxin Response Factor-Auxin Inducible Degron) system is a reengineered auxin-inducible protein degradation system. Inducible degron systems are widely used to specifically and rapidly deplete proteins of interest in cell lines and organisms. An advantage of inducible degradation is that the biological system under study remains intact and functional until perturbation. This feature necessitates that the endogenous levels of the protein are maintained. However, endogenous tagging of genes with AID can result in chronic, auxin-independent proteasome-mediated degradation. The additional expression of the ARF-PB1 domain in the re-engineered ARF-AID system prevents chronic degradation of AID-tagged proteins while preserving rapid degradation of tagged proteins. Here we describe the protocol for engineering human cell lines to implement the ARF-AID system for specific and inducible protein degradation. These methods are adaptable and can be extended from cell lines to organisms.

Attachments

ARF-AID_methods.pdf
3MB

Guidelines

INTRODUCTION:

A diversity of molecular tools that disrupt genes are commonly used to gain mechanistic insight into protein function. Many of the methods available today disrupt gene function by genetic knock-out or RNA degradation. These methods can be universally applied to study most genes and allow us to understand the cumulative effect of gene dysregulation. The major drawbacks of these systems are that the kinetics of protein depletion are slow and chronic, ranging from days to the lifetime of an organism, and often irreversible. This is problematic when studying the mechanistic function of a protein, which is most directly assessed by observing the immediate cellular response to dysregulation. Moreover, chronic gene disruption is not possible for essential genes. Small molecule inhibitors and temperature-sensitive mutations are acute, rapid, and reversible, but unique strategies are needed to target each protein of interest. Inducible degron systems are rapid, reversible, and can be universally applied to any protein.

One strategy to directly manipulate protein stability is to induce interaction with a ubiquitin ligase complex, which will lead to polyubiquitination and proteasomal degradation of the protein (Sakamoto et al., 2001; Schneekloth et al., 2008; Schapira et al., 2019). PROTACs (Proteolysis Targeting Chimeras) are heterobifunctional molecules that promote proximity-mediated polyubiquitination. PROTACs are composed of a moiety that binds to an E3 ubiquitin ligase, such as von Hippel-Lindau (VHL) or cereblon (CRBN), and a small molecule that directly interacts with the protein of interest (Bondeson et al., 2015; Lu et al., 2015; Winter et al., 2015; Schapira et al., 2019). This strategy requires a chemical probe for the protein of interest as starting material, and developing PROTACs for each target is time-consuming and requires medicinal chemistry expertise.

The dTAG system provides a more universal system to specifically target proteins of interest for rapid and inducible ubiquitin-mediated degradation. In the dTAG system the protein of interest is fused to a mutant human FKBPF36V, and a single bifunctional molecule promotes proteasome targeting (Nabet et al., 2018). This system is simple and requires only one genetic manipulation in order to tag the protein with FKBPF36V. However, the degradation rate using this system varies depending on the cell type (Li et al., 2019). Additionally, the amount of the dTAG-13 molecule must be titered based on protein levels in order to avoid saturating each end of the molecule independently and not providing a link between the target and the ubiquitin ligase (Nabet et al., 2018; Li et al., 2019).

Other chemical genetics approaches to targeted protein degradation utilize the exogenous expression of plant-specific E3 ubiquitin ligase adaptor proteins in animals and cell lines. The auxin inducible degron (AID) system was the first heterologous system developed (Nishimura et al., 2009). In this system an auxin molecule interacts with the TIR1 protein, which acts as a ubiquitin ligase adapter. This auxin-induced interaction of AID with the SCF-TIR1 E3 ubiquitin ligase complex causes ubiquitination and degradation of the AID-tagged protein (Nishimura et al., 2009). This was followed by the development of the Jasmonate inducible degron (JID) system. Here, in the presence of jasmonate-isoleucine, proteins tagged with the JAZ degron interact with the F-box containing COI1 and are subsequently degraded (Brosh et al., 2016). Recently, another auxin-sensing F-box protein A. thaliana AFB2 (AtAFB2) has been developed as a promising new degron system (Li et al., 2019). Of the direct protein

degradation technologies, the auxin-inducible degron system is the most robust and most widely used system (Lambrus et al., 2018).

Endogenously tagging genes with AID can result in unwanted chronic degradation in the absence of auxin. Supplementing the AID system with an additional component of the plant's native auxin signaling machinery preserves near-endogenous expression levels of the target protein (Sathyan et al., 2019). The canonical AID system has two components: 1) transport inhibitor response 1 (TIR1), and 2) auxin/indole-3-acetic acid (Aux/IAA or AID) proteins (Nishimura et al., 2009). However, in plants, there is another critical component in the auxin signal transduction system: auxin response transcription factors (ARF). In the absence of auxin, ARF binds to the AID protein and protects it from TIR1-mediated ubiquitination. Upon sensing auxin, TIR1 binds to and ubiquitinates the AID protein, which dissociates from ARF (Dharmasiri et al., 2003, 2005; Gray et al., 2001). Introduction of the ARF-PB1 domain in the re-engineered auxin-inducible degron system (ARF-AID) rescued chronic auxin-independent degradation of AID-tagged proteins and increased the rate of auxin-induced AID-tagged protein degradation (Sathyan et al., 2019). A caveat of any auxin system is that the auxins are aromatic hydrocarbon molecules and indole-3-acetic acid (auxin) can cause changes in expression of aryl hydrocarbon receptor genes (Sathyan et al., 2019). We look forward to mixing and matching the components of newly developed AID systems in order to further refine these tools.

Simultaneous expression of ARF and TIR1 driven by a robust common promoter ensures high expression of these proteins compared to most cellular proteins (Figure 1 and Figure 2). We generated two multi-cistronic plasmids that express both ARF and TIR1 driven by a CMV promoter (Sathyan et al., 2019). A P2A ribosome skipping site separates these two polypeptides during translation (Figure 2A). In our original work, we used a CMV-driven eGFP-ARF to rescue the chronic degradation of AID-tagged proteins (Sathyan et al., 2019). In that context, we found that the rescued AID-tagged proteins degraded faster when treated with auxin (Sathyan et al., 2019). However, when we first expressed ARF-HA-P2A-TIR1 and then tagged proteins with AID in order to preserve protein levels, the degradation rate was slower compared to eGFP-ARF rescued degradation (Figure 3A,C&E). Therefore, we modified the ARF-HA construct to express eGFP-ARF (Addgene #129668). Both eGFP-ARF and ARF-HA preserve endogenous expression of the tagged proteins and promote comparably rapid degradation kinetics (Figure 3).
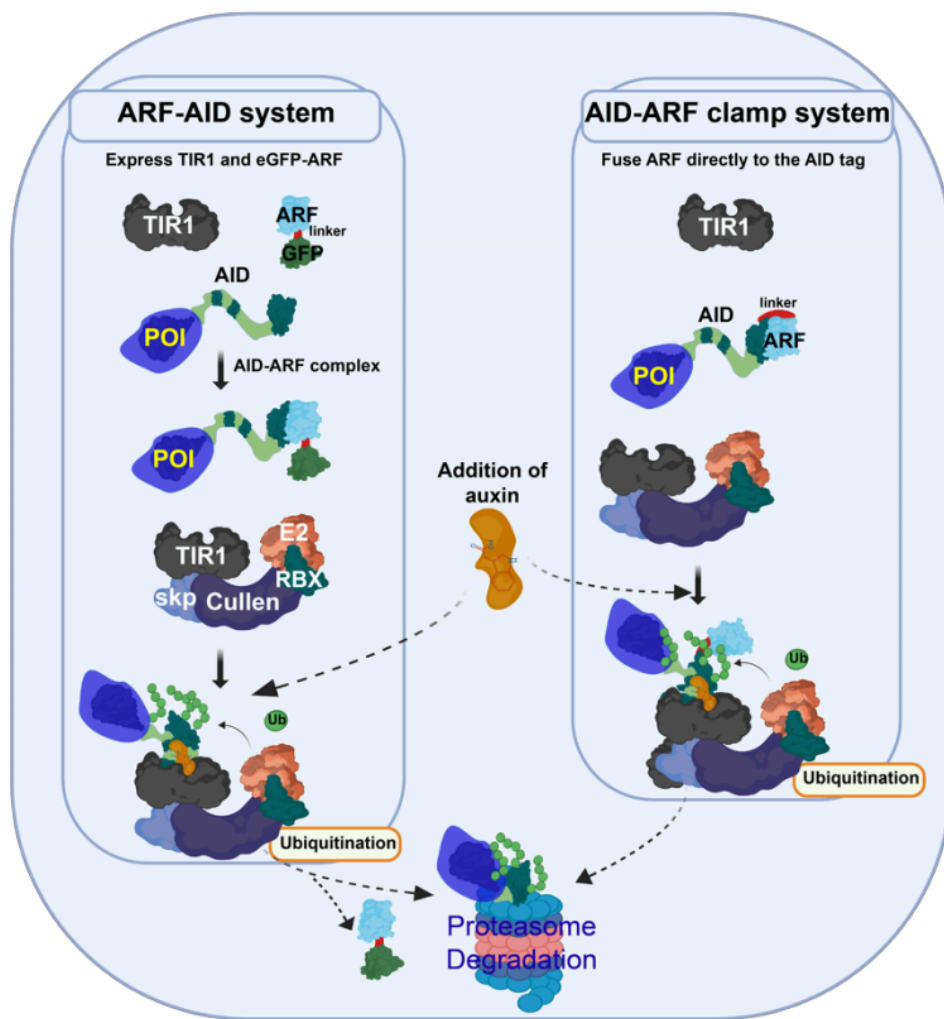
Figure 1. An overview of the components and their roles in engineered ARF-AID systems. The ARF-AID system (left) differs from traditional AID systems due to the presence of the ARF-PB1 domain, which binds to the AID-tag and prevents auxin-independent AID degradation. The AID-ARF clamp system (right) fuses the ARF-PB1 domain to the AID-tag, which also protects AID from auxin-independent degradation. An advantage of the clamp system is that previously generated TIR1-expressing cells and animals can be used as the progenitors for protein tagging. In both systems, auxin facilitates interaction between AID and TIR1 and rapid proteasome-mediated protein degradation.

ARF prevents chronic degradation of the AID-tagged proteins by direct interaction with AID (Sathyan et al., 2019). Therefore, it is important that ARF and the AID-tagged protein are localized to the same subcellular region. The ARF-TIR1 plasmids described here are designed for protein localized in the nucleus, so a nuclear localization signal (NLS) sequence is fused to the ARF-PB1 domain (Sathyan et al., 2019). In order to adopt the system to degrade cytoplasmic proteins, one should replace the NLS with a nuclear export signal.

Stable expression of ARF and TIR1 ensures efficient auxin-inducible degradation of the AID-tagged proteins. Integrating these genes at a safe harbor genetic locus allows ARFPB1 and TIR1 to be stably expressed and resistant to epigenetic silencing (Figure 1 and Figure 2). Virus-mediated integration of the constructs at random genetic loci may lead to variable and unstable expression of ARF and TIR1. We incorporate ARF and TIR1 (eGFP-ARF-P2A-TIR1 or ARF-HA-P2A-TIR1) into the human AAVS1 locus.

Redesigning the eGFP-ARF-P2A-TIR1 plasmid with ROSA26-specific homology arms and using a mouse ROSA26 specific sgRNA (Chu et al., 2016) will allow for integration into mouse cells. For cells from other organisms, design a new sgRNA to the safe harbor locus and eGFP-ARF-P2A-TIR1 or ARF-HA-P2A-TIR1 with corresponding right and left homology arms. The plasmids have approximately 800 nucleotide long homology arms. Shorter homology arms, as few as 30 nucleotides, also permit efficient homology directed repair and have the advantage of increased transfection efficiency (Paix et al., 2017). We recommend generating a clonal progenitor cell line that expresses ARF and TIR1, then using this progenitor to tag proteins of interest (Figure 2A). We provide human-specific codon optimized constructs, but codon optimization is recommended for expression in other organisms.
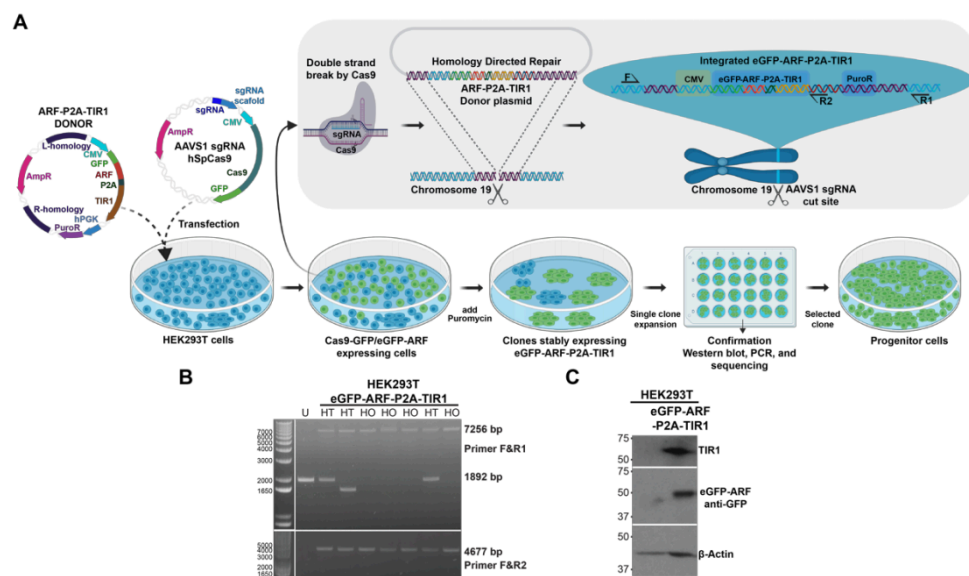

Figure 2. Generating a stable progenitor cell line that expresses the components of the ARF-AID system at a safe harbor locus. A) A step-wise strategy to integrate eGFP-ARF and TIR expressed from a CMV promoter. CRISPR is used to target the multi-cistronic construct into the AAVS1 locus. Primers F and R1 generate a PCR amplicon of 7256bp if the construct is inserted and 1892bp amplicon if the construct is not inserted. R2 primer recognizes a sequence internal to the TIR1 gene, so the primer combination F and R2 amplify only if the construct inserts into the AAVS1 locus. B) Clones show either heterozygous or homozygous integration of the insert. Compare the bands between the unintegrated (U) and integrated clones (HT -Heterozygous, HO -Homozygous). The clone in the fourth lane has one allele integrated and a deletion in the other allele. C) Western blotting confirms that the eGFP-ARF and TIR1 proteins are expressed.

AID-ARF clamp system

All AID systems necessitate the expression of TIR1, so there are many progenitor cell lines and organisms that express TIR1 (Li et al., 2019; Natsume et al., 2016; Nishimura et al., 2009; Zhang et al., 2015; Holland et al., 2012). In an effort to repurpose these cell lines and organisms, but alleviate chronic degradation, we fused the AID-tag with ARF using a flexible linker to create the AID-ARF clamp (Figure 1) (Addgene #138174). We tagged ZNF143 with the AID-ARF clamp using a canonical TIR1 expressing progenitor cell line. Similar to the ARF-AID system, the AID-ARF clamp preserves nearendogenous protein expression (Figure 4A). Moreover, the AID-ARF clamp-tagged ZNF143 protein degraded rapidly upon auxin treatment (Figure 4B&C). Both ZNF143- AID-ARF clones tested showed an average half-life of 10 min (Figure 4C), which is similar to the reported ZNF143-AID degradation half-

life of 11 min in the eGFP-ARF rescued system (Sathyan et al., 2019). In the future, we look forward to testing whether AID-ARF clamp-tagged proteins consistently degrade more rapidly as compared to the canonical AID and multi-cistronic ARF-AID systems

Here we describe methods to implement the ARF-AID system in human cells, which is easily adaptable to other types of cells and for the AtAFB2 system. We will outline a protocol for 1) generation of ARF-TIR1 progenitor cells, 2) tagging the protein of interest with AID, and 3) testing degradation in the tagged cell lines.
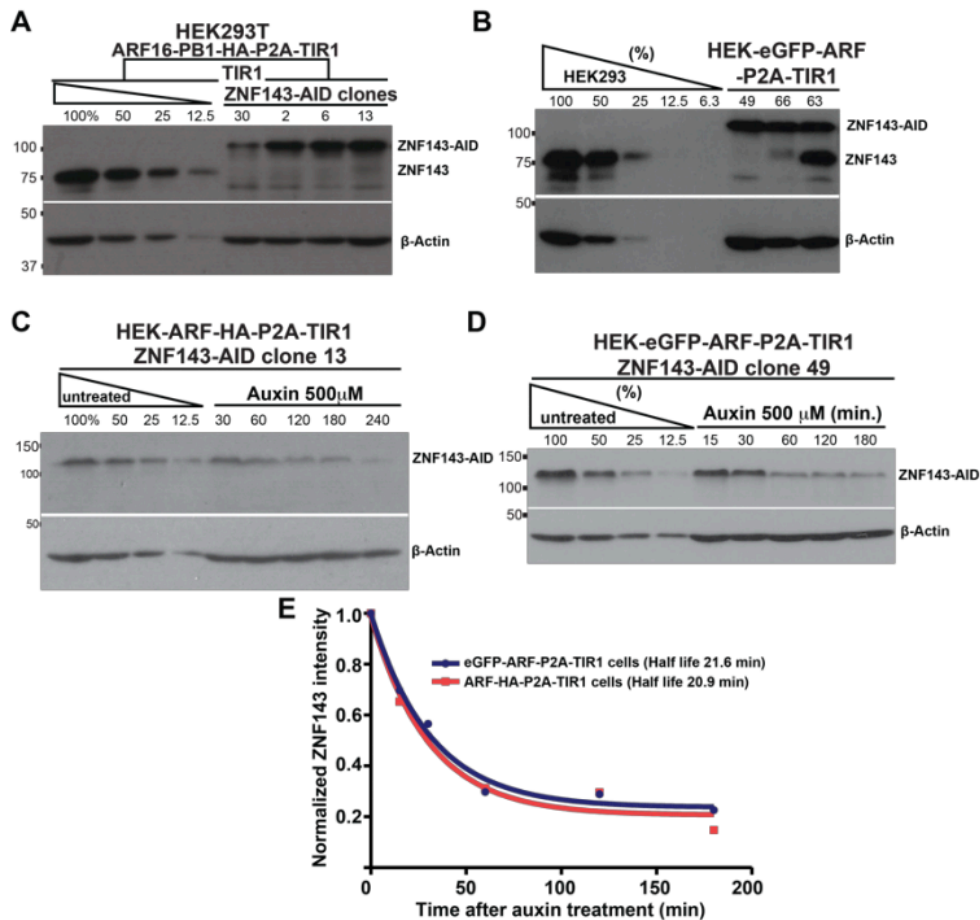

Figure 3. The ARF-AID system preserves the endogenous expression levels of AID-tagged proteins and facilitates auxin-inducible degradation. Tagging of ZNF143 with AID in both an ARF-HA-P2ATIR1 (A) and eGFP-ARF-P2A-TIR1 (B) progenitor line preserved comparable expression levels compared to the progenitor lines. C&D) Both ZNF143-AID cell lines facilitate auxin-inducible ZNF143-AID degradation with (E) protein half lives of approximately 20 minutes.
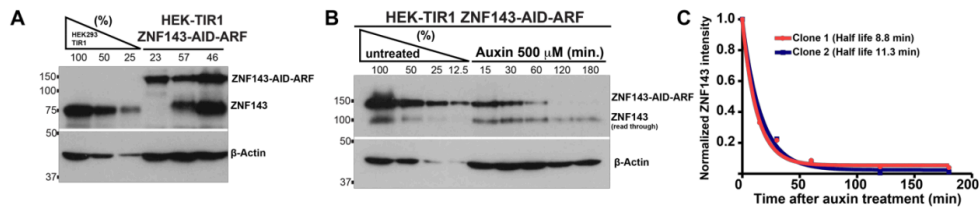
Figure 4. The AID-ARF clamp system facilitates rapid auxin-inducible AID-ARF tagged proteins. A) ZNF143-AID-ARF is expressed at comparable levels to the parental TIR1 expressing cells. B) ZNF143- AID-ARF is rapidly degraded upon addition of auxin with (C) protein half lives of approximately 10 minutes as assessed by two independent clones.

COMMENTARY

TROUBLESHOOTING:

LACK OF POSITIVE COLONIES OR TOO MANY COLONIES DURING TAGGING

If there is difficulty in getting positive colonies, check the efficiency of the sgRNA and try a different sgRNA if the efficiency is very low. Another potential problem is the disruption of the protein function by tagging with the AID. This is true for any essential genes that require both alleles for cell survival or if tagging makes a dominant negative mutant. The rate of homozygous integration is about 10% of the heterozygous integration in HEK293T cell, which has hyper diploid chromosome numbers.

If there are too many colonies and it is difficult to pick individual colonies, then split cells and plate around 100 to 200 cells per 10 cm plate. Depending on the cell type and cell survival after splitting, change the number of seeded cells. Grow cells with conditioned media to help individual cells to form colonies. Approximately 50 colonies in a 10 cm plate is optimal.

TESTING THE FUNCTIONALITY OF THE TAGGED PROTEINS

Absence of any tagged colonies may indicate that the tagged protein is not functional. If you are not able to generate homozygous clones after screening several clones (close to 100 heterozygous clones), then attempt to tag the protein at the other terminus. We recommend tagging a gene such as ZNF143 as a positive control, since ZNF143 is ubiquitously expressed and we previously optimized these sgRNAs and confirmed that C-terminally tagged ZNF143 is functional (Sathyan et al., 2019). To test the functionality of the tagged proteins, initially look at whether the protein localizes to the same compartment as the untagged proteins using immunofluorescence or cell fractionation. The same localization may indicate the protein is functional. For transcription factors, the localization to the same genomic loci is an indication of the functionality of a transcription factor binding and quantitative ChIP-seq can be used to determine if degradation results in genome-wide unidirectional decreases in binding (Guertin et al., 2018). Check the proximity of TF binding and the expression of the regulated genes after treatment with auxin. Depending upon the function of the protein, query the appropriate molecular phenotypes to confirm auxin-induced deficiencies.

TIME CONSIDERATIONS:

There are two components in the canonical AID, ARF-AID, and AID-ARF clamp systems. The generation of a progenitor cell and tagging the protein of interest with the degron. A general outline of the timeline to complete each step for HEK293T cells is given below, which may vary between cell lines used.

ARF-TIR1 or TIR1 progenitor line - 6-8 weeks
Design, clone, and test sgRNAs - 4 weeks.
Design and order the homology-directed repair constructs - 1 week
Tag the gene of interest with AID-tag - 6-8 weeks.

We recommend using established progenitor cells if available to reduce time to tag your protein of interest. Simultaneously developing progenitor cells, testing sgRNAs, and making homology-directed repair constructs significantly reduces the total amount of time to establish degron tagged cell lines.

INTERNET RESOURCES

sgRNA design tools:
Benchling https://www.benchling.com
CHOPCHOP https://chopchop.cbu.uib.no
E-CRISP http://www.e-crisp.org/E-CRISP/
CRISPOR http://crispor.tefor.net

Primer design tools:
Primer3: http://bioinfo.ut.ee/primer3-0.4.0/
IDT: https://www.idtdna.com/pages/tools/primerquest
Benchling: https://www.benchling.com

Troubleshooting

Safety warnings

⛔ Please refer to the Safety Data Sheets (SDS) for safety and environmental hazards.

Before start

STRATEGIC PLANNING

Establishing the ARF-AID system can be time consuming if all steps are performed serially. To reduce time, we recommend developing the ARF-TIR1 progenitor cells in parallel with testing gene-specific sgRNAs and PCR-amplifying the homology-directed repair (HDR) constructs (Figure 5).
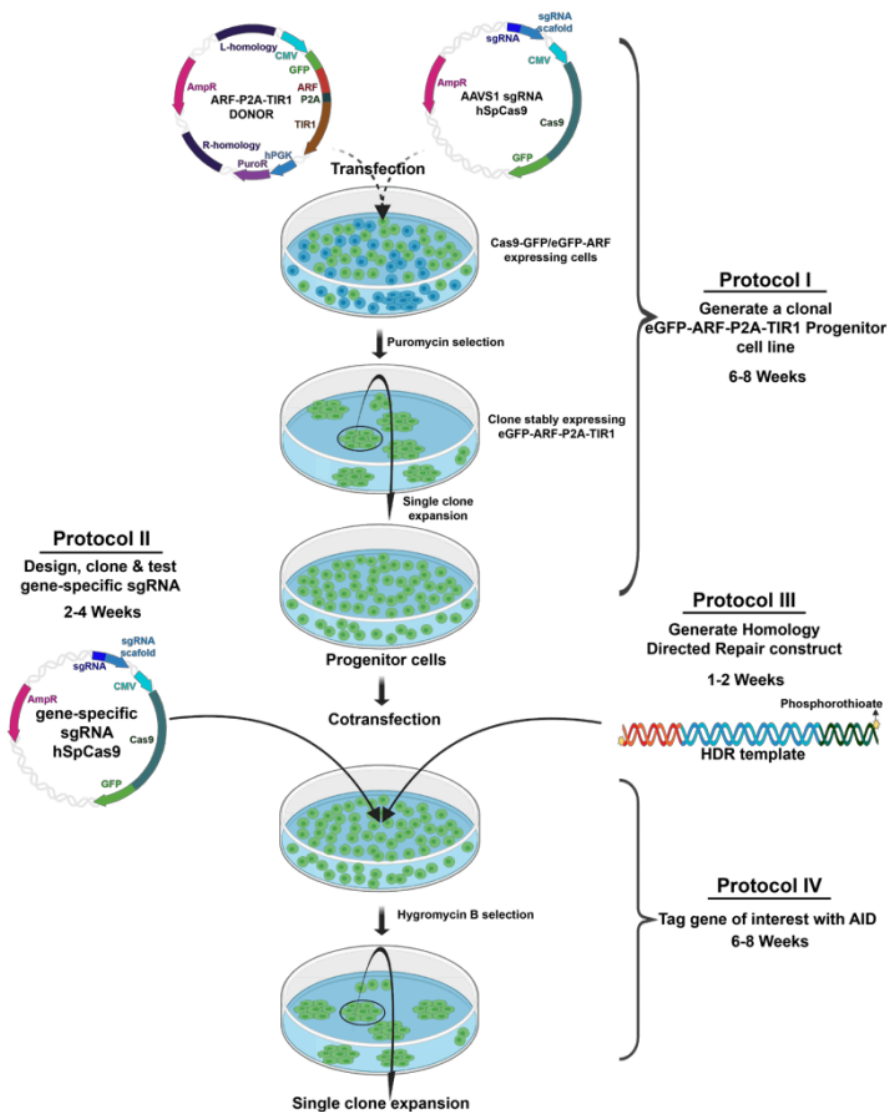

Figure 5. The ARF-AID system has four distinct protocols. Protocol I is used to generate a progenitor cell line that expresses all necessary components of the plant AID system. Protocols I, II, and III can be performed simultaneously. Protocol II involves testing sgRNA cleavage at the gene of interest and Protocol III outlines the design of the homology repair construct. Protocol IV incorporates all these components to tag the protein of interest with AID in the progenitor cell line.

Attachments

[ARF-AID_methods.pd](#)

[ARF-AID_methods.pdf](#)
3MB

Files

SEARCH

Protocol

NAME

Basic Protocol 1: Generation of eGFP-ARF-P2A-TIR1 or ARF-HA-P2A-TIR1 progenitor cells

VERSION 1

CREATED BY

Michael Guertin

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Protocol

NAME

Basic Protocol 2: Tagging a gene of interest with AID

VERSION 1

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Protocol

NAME

Basic Protocol 3: Testing auxin-mediated degradation of the AID-tagged protein

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