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

In 1 collection

Kizhakke Mattada Sathyan¹, Thomas G. Scott¹, Michael J. Guertin^{1,2,3}¹Biochemistry and Molecular Genetics Department, University of Virginia, ²Center for Public Health Genomics, University of Virginia, ³Cancer Center, University of Virginia

1

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Michael Guertin

ABSTRACT

The first procedure for implementing the ARF-AID system is to establish ARF-TIR1 progenitor cells, as shown in Figure 2A. All the plasmids for integrating ARF-TIR1 into the AAVS1 locus in human cells are available from Addgene. The choice of transfection method varies depending on the cell type; lipofectamine 3000 works efficiently for HEK293T cells.

ATTACHMENTS

[ARF-AID_methods.pdf](#)

GUIDELINES

Please refer to the Guidelines & Warnings section of the protocol collection for additional guidelines.

ALTERNATE PROTOCOL 1

Establishment of the AID-ARF clamp system

Materials:

pMGS59 (AID-ARF-P2A-Hygromycin), Addgene# 138174

pMK232 (CMV-OsTIR1-PURO), Addgene [#72834](#)pMGS7 (AAVS1sgRNA) Addgene [#126582](#)

ZNF143 C-terminal targeting sgRNA 5'-GAGGATTAATCATCCAACCC-3'

ZNF143 C-terminal Homology Directed Repair Construct PCR Primers

Forward 5'-

A*A*GAAGCCATCAGAATAGCGTCTAGAATCCAACAAGGAGAAACGCCAGGGCTTGACGACGGTGGATCTGGAGGTTTCAGGTGGCAGTGTCTG
AGCTGAATCT-3'

Reverse 5'-

A*A*GACTCCTTCTGCTTTATTGCTCCATTGTTCTGAGGATTAATCATCCAATCAGTT AGCCTCCCCCATCTC-3'

This method uses the canonical TIR1 progenitor cells without the ARF protein. If the TIR1 expressing progenitor cells are available, directly tag the protein of interest with the AID-ARF clamp. Generate a TIR1 expressing progenitor cell using the TIR1 plasmid developed by the Kanemaki lab (Natsume et al., 2016) (Addgene# 72834) and the sgRNA that targets the AAVS1 locus (Addgene# 126582). Follow the steps 1-1 to 1-44 to make progenitor cells with the exception that this construct does not express eGFPARF.

Tag at the C-terminus of the protein of interest with the AID-ARF clamp using plasmid #138174 from Addgene.

We tagged ZNF143 at the C-terminus with the AID-ARF fusion protein (Figure 4) using the sgRNA and the donor primers given in the reagent list.

To tag protein of interest with ARF-AID clamp in the TIR1 progenitor cells, follow the steps 2-1 to 2-56. The only differences are the progenitor cells (TIR1 as opposed to ARF/TIR1) and the HDR template. Use the AID-ARF-P2A-Hygro plasmid (Addgene # 138174) to generate the HDR template.

For N-terminus tagging, the order of the AID and ARF fusion and linker properties should be empirically determined.

MATERIALS

NAME ▾	CATALOG # ▾	VENDOR ▾
0.05% Trypsin-EDTA (1X)	25300-054	Thermo Fisher Scientific
Lipofectamine 3000	L3000015	Thermo Fisher Scientific
Platinum™ Taq DNA Polymerase High Fidelity	11304011	Thermo Fisher
dNTP Mix (10 mM ea)	18427013	Thermo Fisher
SYBR™ Safe DNA Gel Stain	S33102	Thermo Fisher
Opti-MEM™ I Reduced Serum Medium	31985070	Thermo Fisher Scientific
HEK293T	CRL-3216	ATCC
pMGS46 (ARF16-PB1-HA-P2A-OsTIR1)	126580	addgene
pMGS56 (GFP-ARF16-PB1-P2A-OsTIR1)	129668	addgene
pMGS7 (AAVS1sgRNA)	126582	addgene
DNeasy blood and tissue genomic DNA isolation kit	69504	Qiagen
Puromycin	A11138-03	Gibco - Thermo Fischer
Cloning cylinder	378470100	Bel-Art
Nonfat dry milk	1706404	BioRad Sciences
Anti-OsTIR1 pAb	PD048	Mbl International
Anti-GFP mAb	11814460001	Sigma
Anti-HA11 mAb; 1:1000	901513	BioLegend
Anti-β-Actin mAb; 1:5000	A1978	Sigma
PBS	10010023	Gibco - Thermo Fischer

MATERIALS TEXT

Reagents and Solutions

HEK293T growth media (565 ml)

- DMEM (Gibco 11965-092): 500 ml
- Sodium pyruvate (100mM, Gibco 11360070): 5 ml
- L-Glutamine 100x (Gibco 25030081): 5 ml
- Fetal bovine serum (VWR 89510186): 50 ml
- Pen/Strep (Gibco 15140122): 5 ml

5x SDS buffer (Laemmli buffer) (stored at room temperature)

- 312.5mM Tris HCl pH 6.8
- 10% SDS
- 50% (w/v) glycerol

- 0.05% bromophenol blue

Dilute 5x SDS buffer with water to 2x and add 50 µl 2-mercaptoethanol per ml of 2x SDS sample buffer prior to use. The 2x SDS sample buffer with 2-mercaptoethanol can be stored at -20 °C.

50x Tris Acetate EDTA (TAE) buffer, pH 7.2

- 2M Tris base
- 1M Sodium Acetate
- 50 mM EDTA

pH to 7.2 with acetic acid and store at room temperature. Make 1x working solution by diluting in double distilled water.

SAFETY WARNINGS






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

BEFORE STARTING

Please refer to the Guidelines & Warnings section of the protocol collection for additional information.

Cotransfection of eGFP-ARF-P2A-TIR1 or ARF-HA-P2A-TIR1 and AAVS1 sgRNA

1 

Remove media from the HEK293T cells and wash with PBS. Add  **0.5 ml**  **0.05 %** trypsin to the plate. Incubate the cells for  **00:02:00** to  **00:03:00**, rinse and collect cells with  **10 ml** of media.

2 




Remove trypsin by centrifugation at  **500 x g** for  **00:05:00** using a swinging bucket centrifuge, remove supernatant, and resuspend the cells in  **10 ml** fresh media.

3 Seed $2.0\text{--}3 \times 10^5$ cells per well of a six-well plate to get 30 to 40% confluent cells the next day.




Cotransfect 1 µg each of AAVS1 sgRNA (Addgene # 126582) and eGFPARF-P2A-TIR1 (Addgene # 129668) or ARF-HA-P2A-TIR1 (Addgene # 126582) plasmids with lipofectamine 3000 reagents. Transfection reagents or methods of introducing foreign DNA into cells will vary by cell line. For HEK-293T cells we recommend a ratio of 2.5 µl of lipofectamine for every µg of DNA. As a negative control, transfect the parental vector pX458 with the repair plasmid and include one well as non-transfected control.

4 

Add  **125 µl** Opti-MEM I reduced serum media in two 1.5 ml tubes for each transfection; one tube is labeled with the description of the DNA sample and the other is labeled as Lipofectamine. Add  **5 µl** Lipofectamine 3000 reagent to the tube labeled Lipofectamine. Add  **5 µl** p3000 reagent to the tube labeled DNA sample.


5 

Add  **1 µg** each of AAVS1 sgRNA (Addgene # 126582) and eGFP-ARF-P2A-TIR1 (Addgene # 129668) or ARF-HA-P2A-TIR1 (Addgene # 126580) plasmids into the p3000 reagent mixture and mix by pipetting up and down three times. Transfect *parental vector*pX458 with eGFP-ARF-P2A-TIR1 or ARF-HA-P2A-TIR1 as negative control.





If the transfection efficiency is low, increase the amount of Lipofectamine 3000 and p3000 reagents to 7.5 µl.

6 

Transfer the lipofectamine mixture (step 4) to the DNA/p3000 mixture (step 5) dropwise slowly. This tube contains ~  **260 µl** of transfection reagents, DNA and Opti-MEM. Mix by pipetting up and down three times with the same pipette tip.


7 

Incubate at  **Room temperature** for  **00:15:00** and transfer the DNA complex into the cells dropwise in a serpentine pattern to uniformly distribute the reagent across the plate.

8 


Rock the plate sideways four times and front to back four times to distribute the reagent even over the cells--*do not swirl the plate*. Place cells in the incubator.

9 **Twenty-four hours after transfection**, replace the media with fresh media

10 **Forty-eight hours after transfection** expand each well of the six-well plate into a 10 cm plate with  **10 ml** media as described in steps 1 and 2.




Expanding cells into 10 cm plates spreads colonies ensures that the cells are sufficiently sparse to allow clonal colonies to form in isolation. Transfect multiple wells to ensure that a sufficient number of colonies survive.

11 **Seventy-two hours after transfection**, begin selection by adding  **1 µg/ml** puromycin. Antibiotic selection concentration varies between cell types. We recommend plotting a titration vs. cell viability curve to determine the lowest concentration at which nearly all cells die within 5 days.



If the number of colonies are low, begin selection after 120 hours, as opposed to 72. The addition of conditioned media (8:2 ratio of fresh media to filtered cultured media from the same cell type) increases the survival of the clones.

12 **After three days of selection** replace with fresh media containing puromycin ( **1 µg/ml**) and continue selection for two days.

13 

(optional) **After five total days** if there are many remaining cells in the negative control plate, then replace with fresh media containing puromycin and continue selection for two days for a total of seven days under selection.

14 **After five to seven days under selection** all the cells in the untransfected control well should be dead. Remove selection media and expand cells without selection.





Allow colonies to grow and expand. Usually, it takes 2 to 3 weeks for colonies to appear after beginning the puromycin selection (Figure 2A&5), but the time frame is variable and dependent upon the cell line. Check the plate under a microscope and scan for colonies that appear after two weeks. The presence of a substantially higher number of colonies in the transfected plates compared to the control plasmid-transfected or untransfected plates suggests successful integration of the plasmid. The control sgRNA plasmid transfected plates often contain some colonies, but many fewer.



Picking colonies




15 Mark individual colonies on the bottom of the plate using a marker. Check eGFPARF-P2A-TIR1 cells using a fluorescent microscope to identify GFP-positive colonies. Select colonies in which all the cells in the colony exhibit uniform nuclear expression of GFP. If the plasmid is modified to localize ARF into cytoplasm or uniformly throughout the cell, then mark those with appropriate GFP distribution. In the case of the ARF-HA-P2A-TIR1 transfected cells select all colonies.

16 

Carefully pick individual colonies by taking  10 µl  0.05 % trypsin solution using a P20 pipette set to 20 µl. Alternatively, use cloning cylinders to pick individual colonies.




For hand picking colonies without the use of cloning cylinders: 1) hold the plate at a 45-degree angle to pool the media away from the colonies; 2) use a P20 pipette set to 20 µl and aspirate ~  10 µl of trypsin solution into the pipette tip; 3) dispense a few microliters of the trypsin solution onto the colony such that a small droplet is formed between the plate and the pipette tip, taking care not to dispense so much that the droplet rolls down the plate; 4) scrape the colony with the pipette tip while the trypsin media remains as a bridge between the plate and the tip; and 5) once the colony is dislodged, aspirate the cells into the tip and transfer of the entire colony to the 96 well plate. Resuspend the colonies in  200 µl media and transfer into 24 well plates containing 1 ml media per well.

Using cloning cylinders: 1) dispense silicone grease onto a glass petri dish and autoclave it along with a pair of forceps; 2) aspirate media from the plate and wash with PBS; 3) hold the plate at a 45-degree angle to pool the PBS away from the colonies; 4) use forceps to pick up a cloning cylinder, dip the thicker edge into silicone grease, and place it over the colony; 5) add  20 µl of trypsin solution and incubate at  37 °C until cells begin to detach; 6) resuspend the colony in  100 µl media and transfer into a 24 well plate containing 1 ml media per well.

17 When the cells reach confluency continue to the next step.

Expanding cells, screening for genomic integration of the ARF-TIR1, and Western blotting

18 

Collect cells by pipetting up and down and transfer **100 µl** into a new plate containing media to continue passaging the cells. Transfer the remaining **~0.9 ml** into a 1.5 ml tube.



The plate allows the expansion of the positive colonies. HEK293T cells attach loosely to the plate, so pipetting is enough to dislodge the cells. For other cell lines, collect cells by trypsinization.

19 Aliquot **100 µl** of the **0.9 ml** within the eppendorf tube into a fresh tube for genomic DNA isolation and PCR. Place cells immediately **On ice**.

20 Centrifuge the remaining **0.8 ml** of cells at **6000 x g** for **00:02:00** using a fixed angle rotor table top centrifuge and remove media.

21 

Add **100 µl** 2x SDS sample buffer (Laemmli buffer) and mix thoroughly by pipetting up and down to generate cell lysate for Western blotting.

22 

Heat the lysate at **95 °C** for **00:05:00** on a heating block and vortex for **00:00:10**. Place back on the heating block for another **00:05:00**. Briefly centrifuge the samples at **5000 x g** and store at **-20 °C**.

Screening genomic integration at the AAVS1 locus

23 To screen the integration of ARF-TIR1 at the AAVS1 locus, we used genomic PCR using primers that amplify the integrated plasmid DNA.

Genomic DNA isolation

24 

Centrifuge cells from step 21 at **6000 x g** for **00:02:00** using a fixed angle rotor table top centrifuge, remove media, and either flash freeze or proceed immediately with gDNA isolation.



Use any genomic DNA isolation kit or conventional phenol-chloroform isolation of DNA to isolate DNA from the cells. Here we used the Qiagen genomic DNA isolation kit. The method described here is adapted from the kit manual with minor changes (DNeasy Blood and Tissue Kit, 69504).

25 Resuspend the cells in **200 µl** PBS and add **20 µl** proteinase K.

26 

Lyse cells by adding  **200 µl** Buffer AL and mix thoroughly by vortexing to form a homogenous lysate.

27 

Incubate samples at  **56 °C** for  **00:10:00** .

28 

Add  **200 µl** 100% ethanol and vortex.





29 Transfer the lysate into a DNeasy Mini spin column placed in a 2 ml collection tube.

30 


Spin at max speed in a tabletop centrifuge ( **10000 x g** -  **17000 x g**) for  **00:01:00** and discard the flow-through.

31 

Place the spin column back into the collection tube and add  **500 µl** Buffer AW1. Spin at  **10000 x g** -  **17000 x g** and discard the flow-through.

32 Place the spin column back into the collection tube and wash by adding  **500 µl** Buffer AW2 and centrifuging at  **10000 x g** -  **17000 x g** for  **00:01:00** .

33 

Discard the flow-through and centrifuge again at maximum speed for  **00:02:00** .

34 

Place the spin column into a 1.5 ml tube and add  **100 µl** nuclease-free water to the center of the column. Spin at  **9000 x g** for  **00:02:00** .



Centrifuging at 9000 x g reduces the chance of breaking off the Eppendorf tube's lid.

35 Quantify DNA using NanoDrop and store at  **-20 °C** .






A wide variety of Taq DNA polymerase products are available for PCR. Each may need a slightly different PCR condition. We recommend Platinum Taq DNA Polymerase High Fidelity for validating ARF-TIR1 genomic DNA insertion using PCR. The forward and reverse primers flank the genomic DNA integration site of the ARF-TIR1 construct (Figure 2A). If there is no insert, the PCR produces a smaller product with the flanking primer, but yields a larger product if ARF-TIR1 is properly integrated (details below). Heterozygous integration of the construct results in the two bands. Independently, we also perform a PCR using the flanking forward primer and a primer that is internal to the insert to confirm the integration of the construct at the locus (Figure 2B).

Make a PCR master mix by adding all the components except genomic DNA for the required number of reactions:

PCR reaction mix for one sample:

Genomic DNA (10ng/μl)	5.0 μl
10 μM Primer F	1.0 μl
10 μM Primer R or R 2	1.0 μl
10X High Fidelity buffer	2.5 μl
DMSO	0.5 μl
10 mM DNTP	1.0 μl
50 mM MgSO ₄	1.0 μl
Platinum Taq DNA Polymerase High Fidelity	0.5 μl
Water	12.5 μl

37 

Aliquot  **20 µl** of the master mix into 0.2 ml PCR tubes and add  **5 µl** of  **10 ng/µl** genomic DNA into each reaction mix. Use the parental HEK293T DNA as a negative control.

PCR condition as follows:

Initial denaturation

 **95 °C**  **00:05:00**

30 cycles:

 **95 °C**  **00:00:30**

 **59 °C**  **00:00:30**

 **68 °C**  **00:07:00**

Final extension

 **68 °C**  **00:10:00**

 **4 °C** hold ∞

Primers used

AAVS1GenomicF (F)

5'-CTGCCGTCTCTCTCCTGAGT-3'

AAVS1GenomicR (R)

5'-ACAGTTGGAGGAGAATCCACC-3'

Internal Primer (R2)



5'-ATTATGATCTAGAGTCGCGGC-3'

Electrophoresis

38 Make  **1 %** agarose gel with 1X TAE buffer.

39 Add  **5 µl** 6x DNA sample buffer to the PCR tubes and load onto the 1% agarose gel.

40 Run the samples at constant 90V for  **01:00:00** .

41 Stain the gel with SYBR Safe DNA gel stain diluted at 1:10000 with 1x TAE buffer for  **00:10:00** and wash **twice** with 1x TAE for  **00:10:00** each.

42 

Visualize the bands using a UV transilluminator (Figure 2A&B).



The negative control produces an amplicon of 1892bp with genomic F and R primers. Successful integration of the construct homozygously produces an amplicon of 7256bp, and heterozygous integration shows both amplicons. The PCR using the AAVS1 GenomicF (F) and Internal Primer (R2) primers produce a 4677 bp amplicon only in the integrated cells, not in the negative and control cells (Figure 2A&B).

Confirmation of the clones by Western blotting

43



Integration of the ARF and TIR1 genes at the AAVS1 locus does not necessarily mean the genes are expressed. Use Western blotting to test whether ARF and TIR1 proteins are present in the cell.

Select homozygously integrated ARF/TIR1 clones from the PCR screen.

44 Thaw the frozen protein lysate (Step 25) and heat again at **95 °C** for **00:03:00** to **00:05:00**.

45 Separate proteins by loading **10 µl** of the samples on a **10 %** acrylamide gel, transfer the proteins onto nitrocellulose or PVDF membrane, block membrane with **7.5 %** nonfat dry milk, and probe with anti-GFP or anti-HA and anti-TIR1 antibodies. β -Actin (1:5000; Sigma, A1978) can be used as a loading control. Include parental cell lysate as a negative control (Figure 2C).



After insertion and expression are confirmed by genomic PCR and Western blot, the construct should be sequenced to confirm that mutations were not incorporated during the process. This cell line will serve as the progenitor cell line for tagging genes of interest with full-length AID.



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