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JAX_DPC: Genotyping and selection of whole-gene knockout (KO) and critical exon (CE) deletion edited clones

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William C. Skarnes[1], Enrica Pellegrino[1], Angelina Kendra[2]
[1]Tha Jackson Laboratory; [2]The Jackson Laboratory

MorPhiC Consortium

Enrica Pellegrino
The Jackson Laboratory

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Abstract

This protocol describes a streamlined genotyping workflow tailored for the validation of whole-gene knockout (KO) edited clones. The process begins with crude lysis of duplicate samples, which are archived for long-term storage. Lysates are diluted and amplified using optimized polymerase chain reactions (PCRs) with PrimeSTAR GXL polymerase, ensuring reliable amplification of KO target regions. PCR products are analyzed via agarose gel electrophoresis to confirm successful amplification and are subsequently sequenced using Sanger sequencing for precise identification of knockout edits. This scalable protocol supports processing in 96-well plate formats and is designed for reproducibility across diverse gene-editing projects.

Troubleshooting

Reagent List

1 Reagents

	A	B	C
	Reagent	Vendor	Catalog
	Water, HPLC Plus	Sigma-Aldrich	34877-1L
	TWEEN 20	Sigma-Aldrich	P9416-100ml
	Potassium chloride	Sigma-Aldrich	P9541-500G
	Magnesium chloride solution	Sigma-Aldrich	M1028-100ML
	TERGITOL solution (NP40)	Sigma-Aldrich	NP40S-100ML
	Proteinase K from Tritirachium album	Sigma-Aldrich	P6556-100MG
	PrimeSTAR GXL DNA Polymerase	Takara Biotech	R050B
	Dimethyl sulfoxide	Sigma-Aldrich	D2650-100ML
	TriTrack DNA Loading Dye (6X)	Fisher Scientific	FERR1161
	Agarose LE, Molecular Biology Grade, Ultrapure	Fisher Scientific	16500-500
	Invitrogen SYBR Safe DNA Gel Stain	Fisher Scientific	S33102
	GeneRuler Express DNA Ladder, ready-to-use	Fisher Scientific	FERSM1553
	Invitrogen Tris (1 M), pH 8.0, RNase-free	Fisher Scientific	AM9855G

LABORATORY PROCEDURES

2 Regardless of the editing strategy, the genotyping pipeline (Fig. 1) for full-gene knockouts (KO), critical exon excision (CE), and introduction of a premature codon termination plus frameshift (PTC) begins with crude lysis of samples that have been

duplicated and archived in liquid nitrogen storage. The lysates are then diluted and used for PCRs with the PrimeSTAR GXL polymerase, which has been optimized for use on even the most challenging target regions. PCR products are run on an agarose gel and submitted for Sanger sequencing. Specific combinations of PCRs, gels, and Sanger sequencing enable complex genotyping across various project types. This section provides detailed protocols for processing 96W plates at each of these stages.

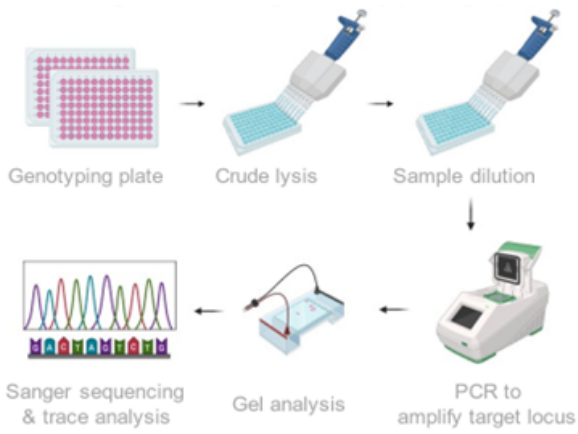


Figure 1. Genotyping pipeline overview.

Lysing cells for PCR genotyping

3 PCR amplification and Sanger sequencing is performed from diluted crude whole cell lysates. The process is simple, efficient, and robust, requiring only a few inexpensive reagents — two detergents that break down lipids (e.g., the cell membrane), proteinase K to degrade endogenous proteins (e.g., DNases and RNases), and some salts to buffer the nucleic acids. This process can be used with either fresh cells or culture plates frozen down to −80°C. A large volume of buffer mix can be prepared at one time (Fig. 2), with detergents and ProK added immediately before use. Note that if plates are already frozen, they should remain in the freezer until the buffer mix and all materials are ready; when they are removed from the freezer, they should be unsealed with buffer mix added immediately.

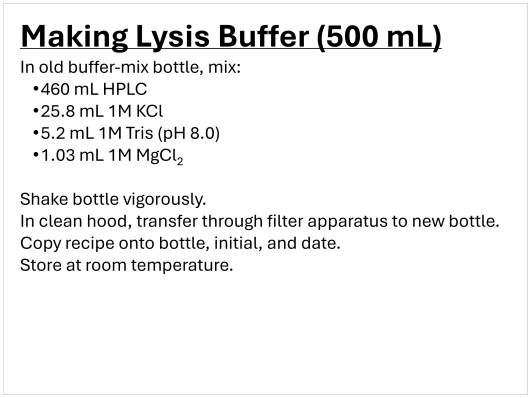
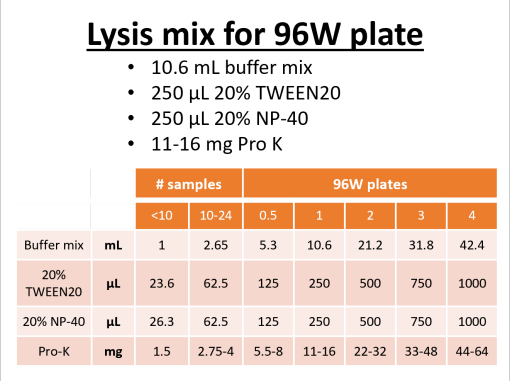


Figure 2. Reagents and directions for preparing 500mL of lysis buffer.

3.1 For each 96W plate to be lysed, mix the necessary volume (Fig. 3) of reagents (buffer mix, TWEEN20, NP-40, and ProK) and invert the tube gently to mix. (Do not vortex – this causes the detergents to foam and the mixture becomes difficult to dispense.)



		# samples		96W plates				
		<10	10-24	0.5	1	2	3	4
Buffer mix	mL	1	2.65	5.3	10.6	21.2	31.8	42.4
20% TWEEN20	µL	23.6	62.5	125	250	500	750	1000
20% NP-40	µL	26.3	62.5	125	250	500	750	1000
Pro-K	mg	1.5	2.75-4	5.5-8	11-16	22-32	33-48	44-64

Figure 3. Reagent volumes used to make lysis mix for various sample sizes

3.2 Pour the lysis mixture into a disposable reservoir, then use a multichannel pipette to add 100 µl of lysis buffer mix to each well, taking care not to let the tips come into contact with the plate. Seal the 96W plate with a foil seal, replace the lid, tape around the edge with autoclave tape, and incubate at 60°C for at least 4 hours.

3.3 After the 4-hour incubation, label a new 96W PCR plate as “gDNA” along with the project title and date (Fig.4, left) for future identification.

Figure 4. Labeling crude lysate, dilution, and PCR plates.

3.4 While the culture plate is still warm from incubation, transfer 100 µL of the lysates to the new gDNA plate, seal, and heat deactivate the proteinase K by incubating the plate at 95°C for 10 minutes in a deep-well thermocycler. This is a critical step to prevent the protease from cleaving the polymerase that will be used in the PCR. The thermocycler can be programmed to hold the lysate at 60°C indefinitely after this 10-minute period (Table 1).

	A	B
	Deactivate	Hold
	– 1 –	– 2 –
	95°C	60°C
	10:00	∞

Table 1. Proteinase K deactivation program for the deep-well thermocycler.

Diluting crude lysates for PCR

4 Label a second PCR plate as “1:10” for future identification (Fig.4, center).

4.1 With a reservoir and a multichannel pipette, add 45 µL of HPLC water to each well of the dilution plate.

4.2 While the gDNA plate is still warm from the thermocycler, transfer 5 µL of each gDNA sample to the dilution plate.

4.3 Reseal the undiluted lysate plate and store at –20°C. DNA is robust and maintains its integrity for long periods of time if frozen; properly stored lysates can be used in PCR

experiments for several years after lysis.

4.4 Seal the dilution plate and store at 4°C for up to a week.

Setting up a PCR experiment

5 PrimeSTAR GXL polymerase (Takara) is designed to use in nearly all conditions for amplifying regions up to 10kb. For projects with an especially high GC content (usually over 65%) increasing the ratio of dimethyl sulfoxide (DMSO) can destabilize those strong triple bonds. Some projects with particularly long target amplicons may require the use of undiluted crude lysates or column-purified DNA extracted from cell cultures or pellets to increase the likelihood of beginning with unfragmented genomic material.

5.1 Label a third PCR plate as “PCR” (Fig. 4, right) for future identification and organization.

5.2 Make a master mix by combining the following components (Table 2) in a 2 µL microcentrifuge tube, then pipet mix gently to homogenize. For a full plate, 108X accounts for loss in pipetting.

	A	B	C	D
	All volumes in µL	**Reagent**	**1x**	**108 x**
	Primer Mix	HPLC	9.5	1026
		PF primer (20 µM)	0.25	27
		PR primer (20 µM)	0.25	27
	Primer Mix per reaction:		*10*	
	PCR Mix	HPLC	1.5	162
		5X PrimeSTAR GXL Buffer	4	432

	A	B	C	D
		dNTP Mixture (2.5 mM each)	1.6	172.8
		DMSO (2.5% final)	0.5	54
		PrimeSTAR GXL DNA Polymerase	0.4	43.2
	Master Mix per reaction:		*8*	
	DNA Template per reaction:		*2*	

Table 2. Reagent volumes needed for setting up a PCR experiment on a full 96W plate.

5.3 Distribute the master mix across 12 strip tubes used as reservoirs for easy multichannel pipetting. (Dispense 150 µL to each tube.)

5.4 Dispense 18 µL of the master mix to each well of your labeled PCR plate.

5.5 Using new tips for each row of samples, use a multichannel pipette to transfer 2 µL of each sample from the 1:10 dilution plate to the PCR plate.

5.6 Seal the dilution plate and return to 4°C storage.

5.7 Seal the PCR plate, vortex briefly, spin down, and put it into the thermocycler.

5.8 Run the GXL program (Table 3), editing the extension time to 1 minute per 1000 bp of your expected amplicon. For example, for an amplicon of 1832 bp, allow 1 minute for the first 1000bp, then add another 50 seconds (0.832∗60s) for the remaining 832bp — adjust the extension time to 1:50. Although the 1kb/minute rate of polymerization is an

estimate and this level of specificity is not strictly necessary, making this quick calculation before running the PCR program is a good reminder to check the settings before finalizing a run. It is safe to leave the plate in the thermocycler on hold for extended periods, such as over a weekend.

	A	B	C	D	E	F
	Denature	Anneal	Elongation	Cycle	Final elongation	Hold
	– 1 –	– 2 –	– 3 –	– 4 –	– 5 –	– 6 –
	98°C	60°C	68°C		68°C	12°C
				GOTO Step 1		
	0:10	0:15	[*adjust*]	44X	2:00	∞

Table 3. Thermocycler settings for a PCR experiment using Takara PrimeSTAR GXL polymerase.

Preparing a gel dilution & loading the gel

6 Gel electrophoresis is an important step in our genotyping pipeline, playing different roles for distinct project types. For knockout editing, you only need to visualize a few representative samples on a gel simply to confirm that the PCR ran successfully and the product is the expected size. For these types of projects, the general procedure is to run out Row A (or Column 1, depending on the plate layout), then submit all samples for Sanger sequencing. Depending on your project, the size of your gel and the number of wells you need will differ. General instructions for pouring a large 1% agarose gel for a full 96W plate are provided below (Fig.5). For a full 96W plate, pour a large gel with four 26W combs. It will take approximately 20 minutes for the gel to polymerize, so begin diluting your samples at that time.

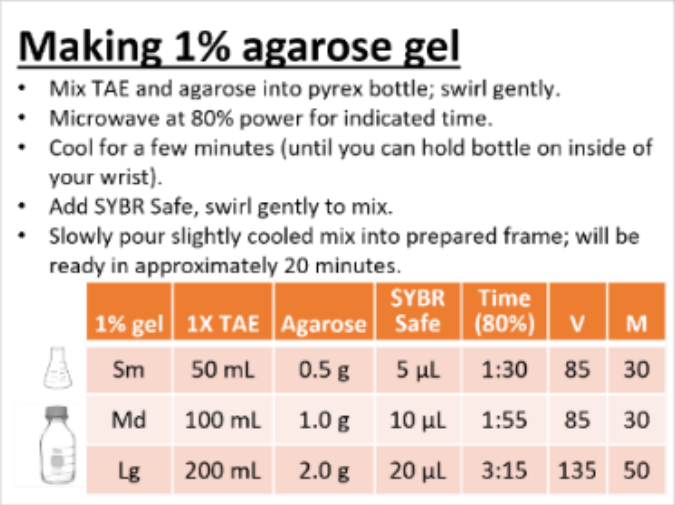


Figure 5. Instructions for pouring a 1% agarose gel.

6.1 Dilute enough 6X loading dye for the full plate by combining 735 µL HPLC and 210 µL 6X dye in a 1.5 mL microcentrifuge tube. Vortex briefly, spin down briefly, then dispense this dilution across 12 strip tubes used as reservoirs for easy multichannel pipetting. (Dispense 75 µL to each tube.)

6.2 Transfer 9 µL of the diluted loading dye to each well of a dilution plate.

6.3 Transfer 3 µL of each sample from the PCR plate to the dilution plate. Pipet mix 2–3X to combine the sample with the dye.

6.4 Add 10.5 µL of DNA ladder to the first well of each gel row. Carefully load 10.5 µL of each prepared sample from your dilution plate to each well of the gel. To load a full plate, we use a 12-channel pipetter and load Rows A and B alternating across the top row of the gel (A1·B1·A2·B2·C3·C3 etc.) Add DNA ladder to the last well of each row. Rows C and D alternate in the second row, E and F in the third, and G and H in the fourth.

6.5 Set the appropriate voltage and time for your gel, usually 120-140V for about 50 minutes. Refer to the table above for suggested settings.

6.6 Image the gel; give it a descriptive name and save it for easy future identification and organization.

ANALYSIS PROCEDURES

7 This section describes the analysis strategies used for genotyping whole-gene knockout (KO) and critical exon (CE) deletion projects, beginning with analysis of pooled samples after each nucleofection, to ensuring that individual clones contain the target edit with no unintended effects.

Genotyping whole-gene knockout (KO) projects

8 Regardless of the size of the gene, knockout projects begin with two “PAM-out” CRISPR guides in the 5′ and 3′ UTRs (Fig. 6), and a donor template that shares 50 bases of homology with the remaining sequence of the UTRs (Fig. 7). At the center of this bridging oligo, the two native bases flanking the deletion are replaced by degenerate bases ordered through IDT using the mixed base codes recognized by the International Union of Biochemistry (IUB).

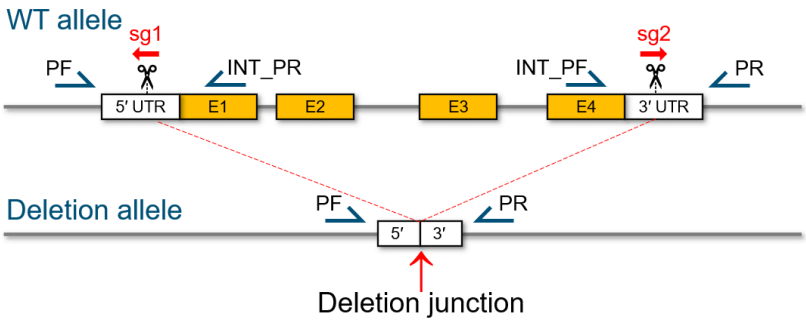


Figure 6. Design of a gene knockout project.

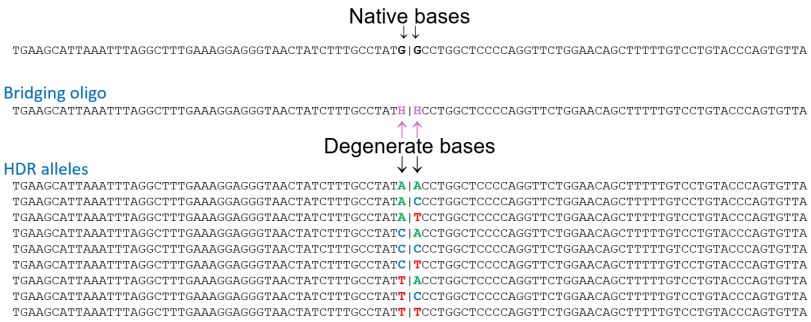


Figure 7. Mixed bases that differ from the native sequence are coded into the bridging oligo at the two positions immediately flanking the deletion junction. A bridging oligo designed with two bases of 3X degeneracy has nine unique combinations at the deletion junction.

9 **Whole-gene knockout (KO) pool analysis**

The first step in assessing the success of a knockout project is to run a PCR on the pooled cells (and unedited KOLF cells as a control), and to run those products on a gel. The PCR primers for knockout projects are designed to sit outside the homology arms of the donor oligo (Fig.8) — this ensures that that the amplicon captures any errors in the HDR process.

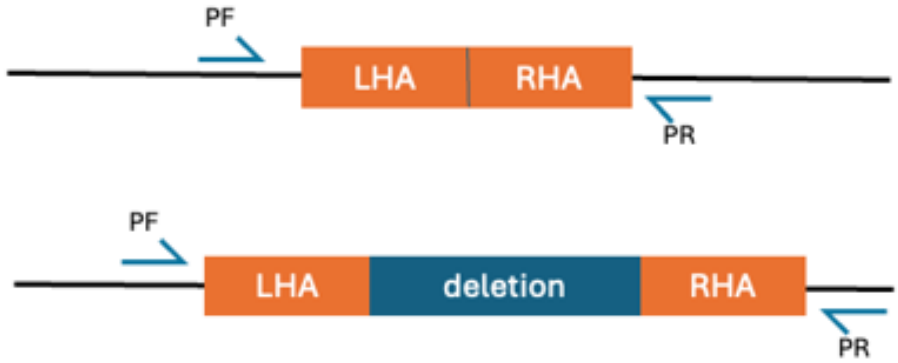


Figure 8. PCR primers are designed outside the donor homology arms.

PCR products of pooled samples are not sequenced — gel imaging is sufficient to determine if the pool contains any knockout alleles. For deletions larger than 5kb, WT alleles will not amplify under standard PCR conditions; for smaller deletions, a WT band may be visible in the control sample and sometimes in the pool, a visible contrast to the smaller target knockout band (Fig. 9). In some cases, if the deletion is small enough and the WT and KO amplicons are roughly the same size, a third intermediate gel band may appear in the sample pool — this is a PCR artifact generated when the larger WT amplicon forms a heteroduplex with the smaller KO amplicon during the final cycle. The mismatched deletion bases on the WT strand bulge in the middle causing the heteroduplex to migrate through the gel at an intermediate rate.

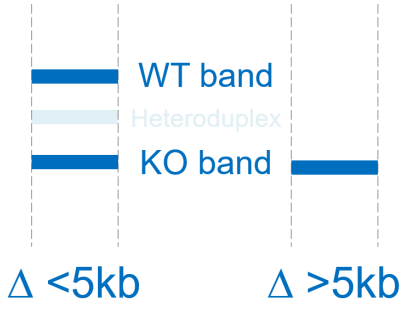


Figure 9. PCR of knockout pools will amplify only the deletion allele, except for deletions smaller than 5kb.

10 **Primary screening for knockouts**

For projects that pass pool genotyping, the standard processes of plating cells, picking colonies, lysis, and dilution are followed by primary (PF|PR) PCR screening of individual clones. Gel electrophoresis cannot differentiate between HET and HOM clones, so the purpose of running knockout PCR products on a gel is simply to confirm that the reaction was successful. If at least one KO band is present, then all samples can be submitted for Sanger sequencing.

When knockout clones are sequenced across the deletion junction, HDR and NHEJ strand repair can be distinguished from the degeneracy designed into the bridging oligo, and the number of distinct peaks at any position differentiates mono- and biallelic knockout clones. There are nine different oligos in the mixture ordered from IDT (Fig. 7), and the native sequence of a clean NHEJ repair may also be evident in traces. Mixed peaks at the deletion junction (Fig. 10) indicate biallelic deletion. There is a one in nine chance that two identical oligos will integrate to a single clone's alleles; those cases may be misclassified as monoallelic (HET) knockouts, but follow-up internal testing (described below) is designed to catch these potential misclassification errors.

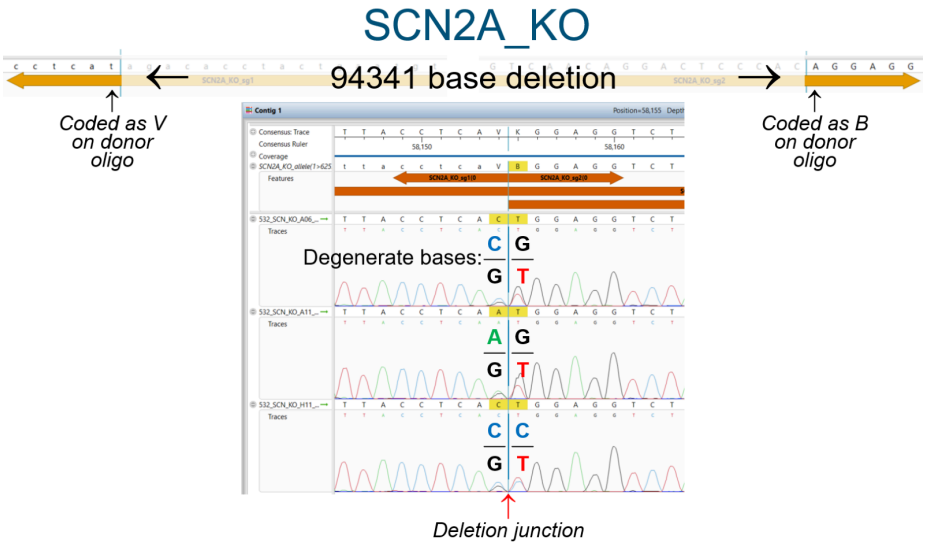


Figure 10. Degenerate mixed peaks at the deletion junction for three AB1 traces for the 94kb SCN2A knockout; these three clones are genotyped as homozygous (HOM) knockouts, via HDR.

Single peaks along the deletion junction indicate monoallelic (HET) deletions (Fig. 11). Any combination of the degenerate bases flanking the deletion junction indicate HDR

repair for that allele. The presence of native bases indicates clean (no indel) NHEJ repair.

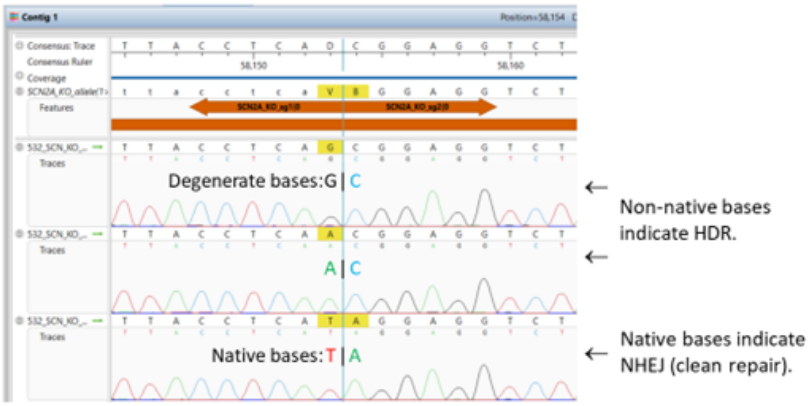


Figure 11. For the same 94 kb SCN2A KO, single peaks represent heterozygous (HET) knockouts. The upper two have degeneracy at the deletion junction indicating HDR; native bases in the lowest trace indicate clean NHEJ repair.

Alleles that are repaired via NHEJ are not always repaired cleanly; insertions or deletions of multiple (sometimes many) bases are common. These can be recognized in sequence alignments by the presence of the misaligned base indicator (—) in either the reference or the sample sequences (Fig. 12). For gene knockout projects, indels do not automatically indicate a failure. If the target deletion is large (the SCN2A knockout design, for example, deletes 94,341 bases), a handful of extra or fewer bases is not functionally problematic.

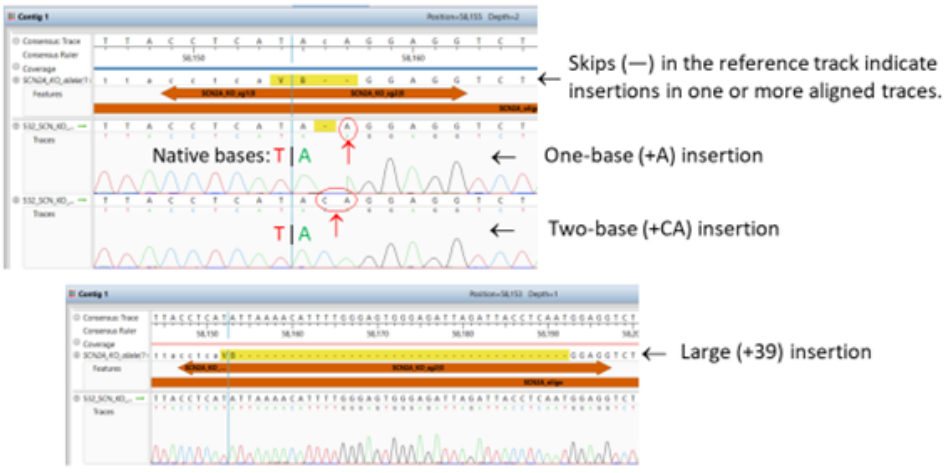


Figure 12. Indel events are flagged with the — symbol in alignments. Especially for large deletions such as this one (94 kb), small indels are still genotyped as knockout alleles. These traces all show HET KOs repaired with NHEJ, with insertions of different sizes.

For most projects, HDR deletion alleles are readily identified; some projects, however, generate large numbers of traces with mixed peaks extending beyond the cutsite (Fig. 13). By closely inspecting each position and looking for stretches of the expected reference sequence, it is possible to manually sort out the two alleles.

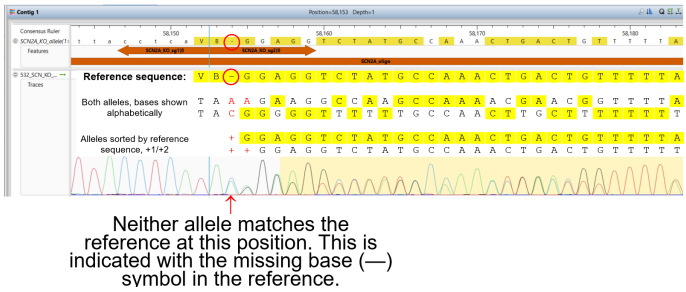


Figure 13. Mixed peaks after the deletion junction represent two knockout alleles with different repair.

11 Internal PCR of knockout clones

After identifying homozygous knockouts with sequence analysis across the deletion junction, it is critical to rule out the possibility of misclassification, contamination, or inversion events before selecting leads for expansion and distribution. This is accomplished by designing an additional pair of primers that sit inside the deleted region (Fig. 14a).

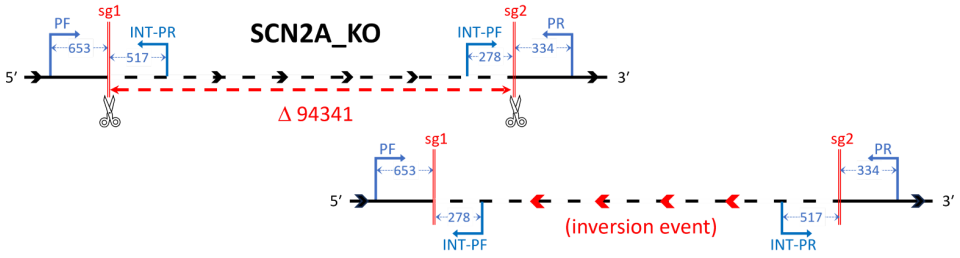
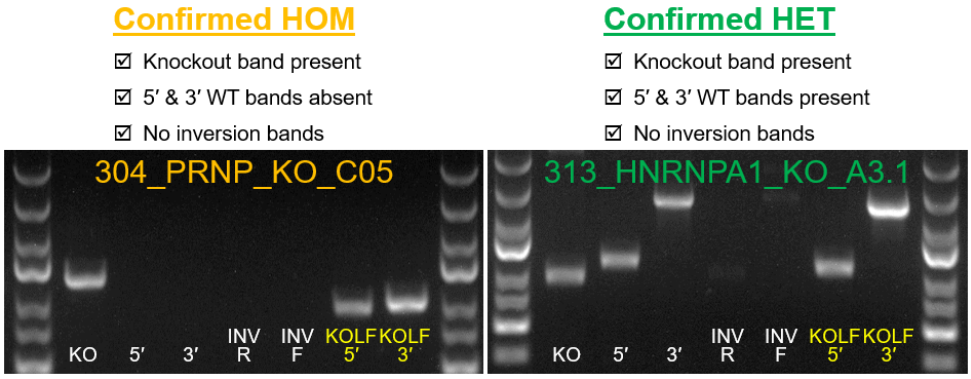


Figure 14a. Design of PCR primers for KO projects.

Band	Primers	Amplicon size (bp)
KO allele	PF|PR	987 *(653 + 334)*
5′ WT segment	PF|INT-PR	1170 *(653 + 517)*
3′ WT segment	INT-PF|PR	612 *(278 + 334)*
Inversion from 5′ end	PF|INT-PF	941 *(653 + 278)*
Inversion from 3′ end	PR|INT-PR	851 *(334 + 517)*

Figure 14b. PCR primers internal to the deletion region identify WT sequence or unintended inversion events.

A series of five PCRs using different combinations of four primers (Fig. 14b) confirms the presence of the deletion on one or both alleles, tests for presence of WT sequence or an inversion event at both the 5′ and 3′ cutsites. For HOM clones, there should be no evidence of 5′ and 3′ WT bands; for HET clones, both WT bands should be present. Control KOLF samples are run with the 5′ and 3′ WT primers to confirm the expected band sizes (Fig. 15). Any indication of an inversion band from either direction fails that clone; there is no control for inversion events.



Band	Primers	Amplicon size (bp)
KO	PF | PR	1267
5′	PF | INT_PR	943
3′	INT_PF | PR	1006
INV-R	INT_PR | PR	1220
INV-F	INT_PF | PF	729

Band	Primers	Amplicon size (bp)
KO	PF | PR	931
5′	PF | INT_PR	1074
3′	INT_PF | PR	2366
INV-R	INT_PR | PR	963
INV-F	INT_PF | PF	2477

Figure 15. Complete internal PCR primer tests for WT sequence and inversions.

Genotyping critical-exon excision (CE) projects

12 As with full-gene knockout projects, CE projects begin with two “PAM-out” CRISPR guides surrounding the area to be removed (Fig. 16), and a donor template that shares 50 bases of homology with the remaining sequence of the UTRs (Fig. 7). The CE bridging

oligo is designed just like a KO bridging oligo, with the two native bases flanking the deletion replaced by degenerate bases.

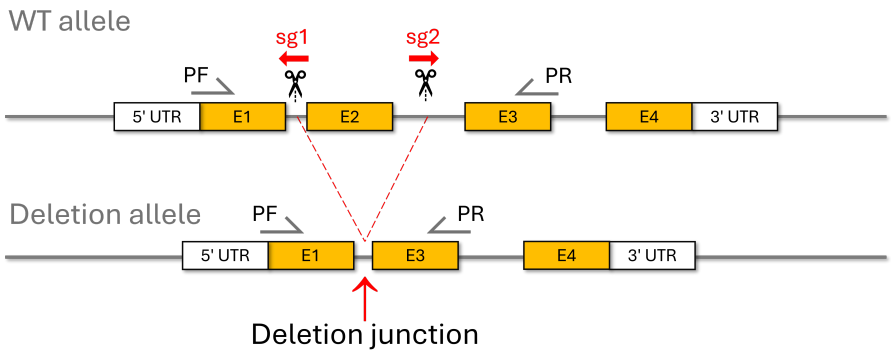


Figure 16. Design of a critical exon excision project.

13 **Critical exon pool analysis**

The first step in assessing the success of a knockout project is to run a PCR on the pooled cells (and unedited KOLF cells as a control), and to run those products on a gel. The PCR primers for CE projects are designed to sit outside the homology arms of the donor oligo (Fig. 8) — this ensures that that the amplicon captures any errors in the HDR process.

PCR products of pooled samples are not sequenced — gel imaging is sufficient to determine if the pool contains any knockout alleles. Because most CE projects delete substantially less than 5kb, WT alleles easily amplify under standard PCR conditions; thus, we expect to see a WT band in the control sample and usually in the pool. The target knockout band, however, will be smaller. If this lower band is visible in the pool, we generally also see a third intermediate gel band — this is a PCR artifact generated when the larger WT amplicon forms a heteroduplex with the smaller KO amplicon during the final PCR cycle (Fig. 17). The mismatched deletion bases on the WT strand bulge in the middle causing the heteroduplex to migrate through the gel at an intermediate rate.

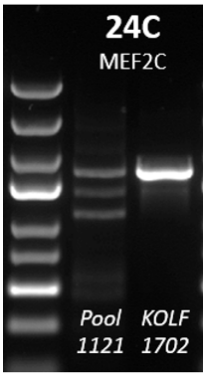


Figure 17. PCR of CE pools will amplify both the WT and deletion alleles.

14 **Primary screening for critical exon excision clones**

For projects that pass pool genotyping, the standard processes of plating cells, picking colonies, lysis, and dilution are followed by primary (PF|PR) PCR screening of individual clones. In contrast to KO projects, gel electrophoresis is an important step to differentiate between HET and HOM clones in CE projects. Following gel imaging, only samples that contain exclusively the smaller deletion band (not combined with the WT band or the possible heteroduplex band) are submitted for Sanger sequencing. When CE clones are sequenced across the deletion junction, HDR and NHEJ strand repair can be distinguished from the degeneracy designed into the bridging oligo, and the number of distinct peaks at any position differentiates mono- and biallelic knockout clones. There are nine different oligos in the mixture ordered from IDT (Fig. 7), and the native sequence of a clean NHEJ repair may also be evident in traces. Mixed peaks at the deletion junction indicate biallelic deletion. There is a one in nine chance that two identical oligos will integrate to a single clone's alleles; for CE clones with a single deletion-band on the gel, single-peak traces are considered to be possible loss of heterozygosity, and are not expanded for further phenotyping or distribution. Occasionally projects with low guide efficiency or HDR require us to deconvolute mixed traces; small indels can sometimes be identified and as long as the exon in question is completely excised that clone can still meet the null criteria.