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JAX_DPC: Genotyping and selection of PTC+1 knockout edited clones

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

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We use this protocol and it's working

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Abstract

This protocol outlines a streamlined workflow for engineering a premature termination codon plus the insertion of one degenerate nucleotide (PTC+1) in human induced pluripotent stem cells (iPSCs) using Cas9 ribonucleoprotein (RNP)-mediated homology-directed repair (HDR). The process begins with the design of a PTC+1 allele, introducing a stop codon (TAA or TGA) in an early exon common to all transcript isoforms, positioned at least 35 amino acids downstream of the start codon to prevent re-initiation of translation. A degenerate base (N) is added immediately following the stop codon to avoid creating novel splice donor sites. Sense strand-specific guide RNAs with minimal off-target effects are selected, and 100-mer asymmetric antisense strand oligonucleotide donors are used for HDR. Following editing, iPSC clones are collected, subjected to crude lysis, and duplicate samples are archived for long-term storage. Lysates are diluted and amplified using optimized polymerase chain reactions (PCR) with PrimeSTAR GXL polymerase and primers pairs to target the edited regions. PCR products are analyzed by agarose gel electrophoresis to confirm successful amplification and are subsequently purified for Sanger sequencing to precisely identify PTC+1 edits. This scalable protocol supports processing in 96-well plate formats and is designed for reproducibility across diverse gene-editing projects, providing a reliable strategy for generating gene-disrupting PTC+1 mutations in iPSCs to facilitate functional genomic studies and disease modeling.

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.

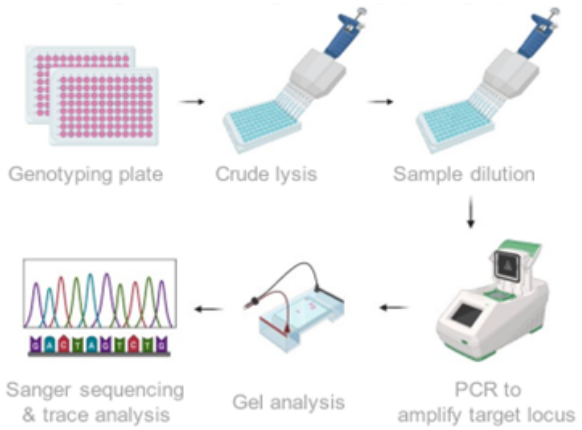


Figure 1. Genotyping pipeline overview.

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.

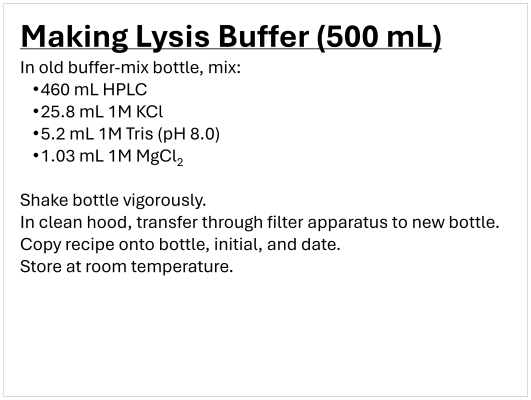
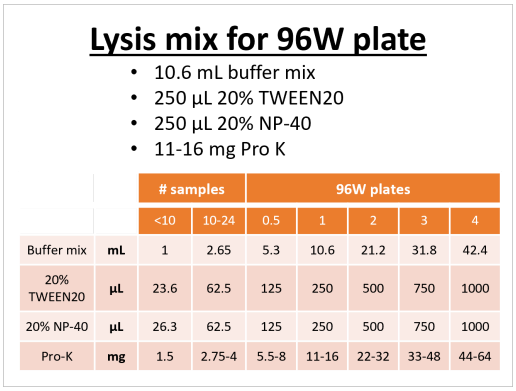


Figure 2. Reagents and directions for preparing 500mL of lysis buffer.

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.

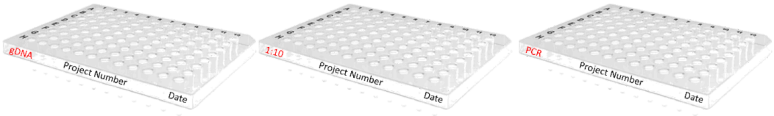


Figure 4. Labeling crude lysate, dilution, and PCR plates.

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
		dNTP Mixture (2.5 mM each)	1.6	172.8

	A	B	C	D
		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.

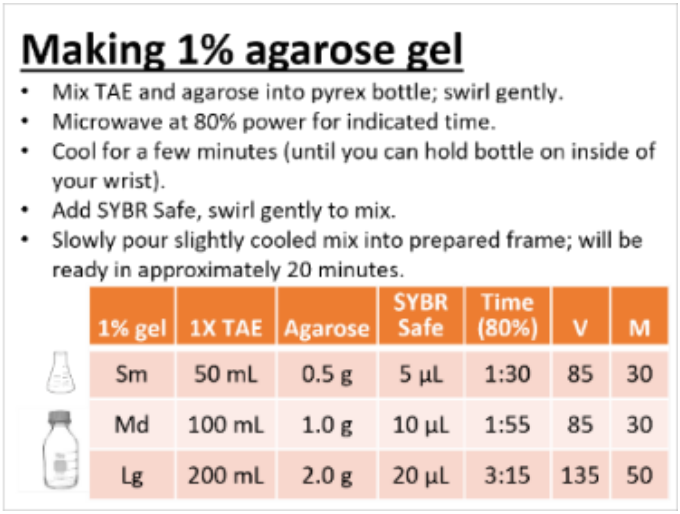


Figure 5. Instructions for pouring a 1% agarose gel.

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 Premature Termination Codon plus frameshift (PTC) projects, beginning with analysis of pooled samples after each nucleofection (Figure 6), to ensuring that individual clones contain the target edit with no unintended effects.

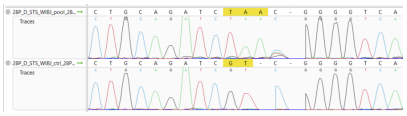


Figure 6. Genotyping premature termination codon plus frameshift (PTC) projects

Genotyping PTC+1 knockout projects

8 For all PTC projects, we begin by lysing a copy of the archived pool followed by amplifying the target exon using PCR primers designed to sit outside the sequence of donor template oligo to ensure that the amplicon captures potential errors in the HDR process. The amplified pool is submitted for Sanger sequencing, and analyzing the traces indicates how effective the editing experiment was. Ideally, we hope to see a trace that is dominated by the termination codon and the frameshifting N base at the original PAM (Fig. 7), but even if the PTC is represented by peaks much lower than the wild-type sequence (Fig. 8), that is sufficient evidence to proceed with screening for edited clones.

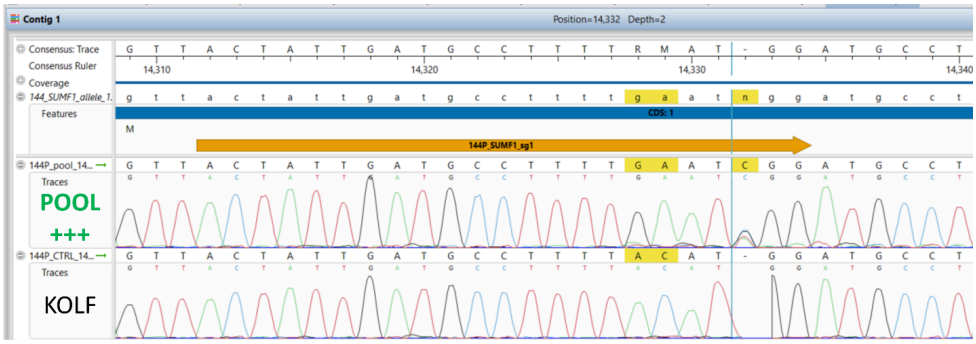


Figure 7. The PTC (in this case “TGA”) peaks should be dominant in the Sanger sequencing traces of the pool.

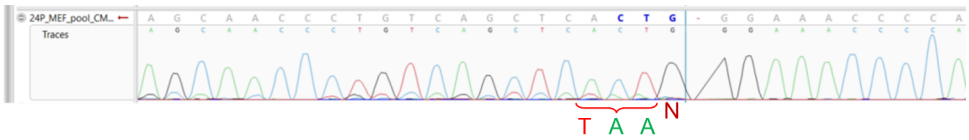


Figure 8. Sanger sequencing traces of a nucleofection pool showing weak – but present – evidence of editing.

9 Primary screening for PTC 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. Gel electrophoresis of a few samples allows us to be certain that amplification occurred correctly before submitting all samples for Sanger sequencing.
When PTC clones are sequenced across the target edit, any signs of mixed peaks beyond the targeted CRISPR cutsite indicates either NHEJ or monoallelic editing (Fig. 9, d). Certain NHEJ events may be sufficient to prevent the transcription and translation of that gene's product, but the design of our template oligo is intended as a more precise method for assuring this. It is also possible to deconvolve mixed-peak traces to identify the presence of WT/PTC heterozygous clones, but monoallelic editing is not the target for MorPhiC projects, so we classify all such clones in a vague "mixed-peak" category. When a clone has clean, single peaks across the target region, we can easily distinguish between unedited (WT) clones because they have neither the PTC nor the frameshifting added base (Fig. 9, c); these align perfectly to the reference sequence for that locus. Edited clones, however, differ from the reference both at the codon that we have targeted to change to the PTC, and by the frameshifting base inserted at the original CRISPR PAM.

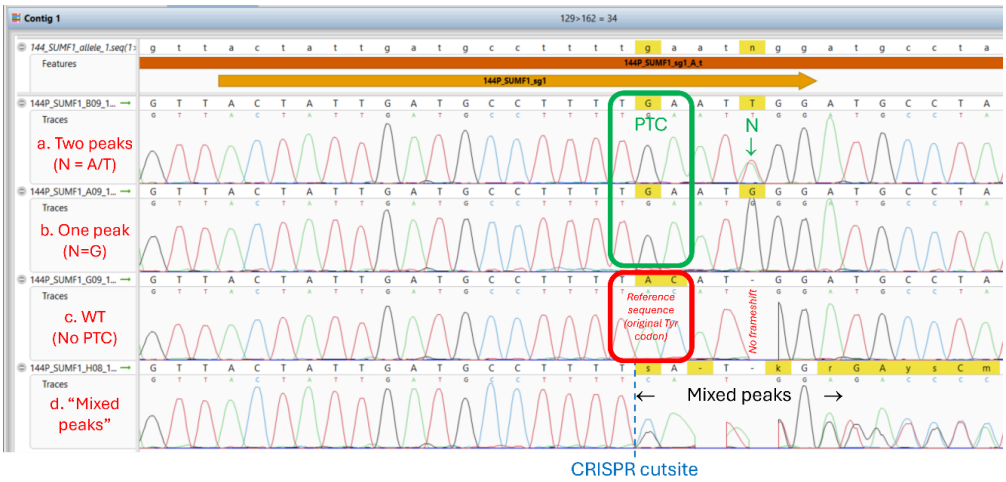


Figure 9. Sanger sequencing can distinguish between clones that have (a) biallelic HDR, (b) biallelic HDR with the same +N base or possible LOH, (c) unedited wild type, and (d) NHEJ or monoallelic HDR.

When we order our donor template oligos from IDT, we use the "any base" code of N at the frameshifting insertion, so there are actually four different oligos in the mixture (Fig. 10). In the case of biallelic editing, we look for clones that show a double peak ONLY at the +N base, indicating that HDR has been completed on two unique alleles (Fig. 9, a).

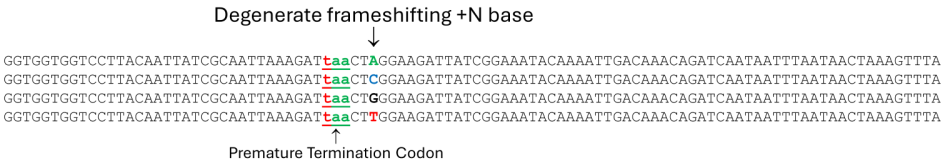


Figure 10. Degeneracy designed into the donor oligo templates can be used as a "barcode" to identify biallelic editing.

Because each allele independently incorporates one of these sequences, we expect 25% of homozygous clones to have the same base at the +N position (Fig. 9, b). Our team classifies these as "likely biallelic" but they could be clones that have experienced a loss of heterozygosity. It is possible to ascertain the presence of two separate edited alleles by amplifying and sequencing known heterozygous SNPs in both directions from the target site using LR-PCR, but in most cases our editing efficiencies are high enough that we have a sufficient number of clones with two different peaks at the +N site so we don't typically complete this assay.