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Southern blot V.1

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Oriol Busquets[1,2], Frank Soldner[1,2]

[1]Albert Einstein College of Medicine, 1301 Morris Park Ave., Bronx, NY 10461, USA.;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 2081

Oriol Busquets
Albert Einstein College of Medicine

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We use this protocol and it's working

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Abstract

This protocol describes a southern blot analysis adapted from standard protocols (https://cshprotocols.cshlp.org/content/2021/7/pdb.top100396#cited-by) to validate the structural integrity and exclude the loss of heterozygosity (LOH) at a genomic locus of interest (PRKN, DJ1, FBXO7 and SYNJ1) following CRISPR/Cas9 or prime editing (PE) based genome editing experiments in human pluripotent stem cells.

Protocol Overview

A. Buffers
B. Generation of Southern blot probes
C. Cell lysis, gDNA extraction and purification
D. gDNA digestion
E. Agarose gel electrophoresis and transfer
F. Membrane blocking and probing
G. Wash and exposure
H. Exposition

Initial Comments

This protocol requires the use of radiolabeled nucleotides and consequently, requires the handling of radioactive materials. Follow your institution's training, regulations and procedures to ensure proper handling of these hazardous materials and ensure researcher's safety.

A list of reagents and relevant vendor information can be found in the table listed under the materials tab.

Oligonucleotide sequence table

	Purpose	Sequence	Restriction Digest
	Detection of PRKN X3DEL mutation / zygosity check (1)	CAGCCTGAGACCATTCCTCT	PvuII
		AGTGTGACATGCTTGCGTTC	
	Detection of PRKN X3DEL mutation / zygosity check (2)	GGCGATATCAGATGTGGGGA	PvuII
		AATGTCCCTCGAACAGTGGT	
	Detection of DJ1 X1-5DEL mutation / zygosity check (1)	AGTGCAGAGTACAG GCG TT	NdeI or AseI
		ACCCACAAGCTACT AGACC	
	Detection of DJ1 X1-5DEL mutation / zygosity check (2)	ACATGGGCTTTTCTATATCTGC	NdeI or AseI
		ACCTCCATTCATCATTTTGTCT	
	Detection of FBXO7 FS mutation / zygosity check (1)	TGTTGACTATGTGGAGAAAGCC	EcoNI or NdeI
		GGGACACAGGCACAGGTATA	
	Detection of SYNJ1 R258Q/FS mutation / zygosity check (1)	ACTCTTAGGTGTTCTGCGGT	SapI
		ACTCTTAGGTGTTCTGCGGT	

	Purpose	Sequence	Restriction Digest
	Detection of SYNJ1 R258Q/FS mutation / zygosity check (2)	CCTGTATAGTGGCCTGTTTTGG	SphI
		AGCAGCTCAATTTCAATGGCT	
	Detection of SYNJ1 R258Q/FS mutation / zygosity check (3)	AAGGTGGAGGGTGGAAATTC	SphI
		GACCCACACTTCCAGCACTA	

Attachments

Southern blot.pdf

92KB

Materials

	Item	Vendor	Catalog Number
	AccuPrime‰ Taq DNA Polymerase, high fidelity	ThermoFisher	12346086
	E.Z.N.A.‰ Gel Extraction Kit by Omega Bio-Tek	Omega Bio-Tek	D5000
	Tris base	Fisher	BP152
	EDTA	Fisher	BP120
	NaCl	Fisher	BP358
	SDS	Thermo	41953
	Proteinase K	Sigma	P2308
	Isopropanol	Fisher	A416
	Ethanol	Decon Labs	DSP-MD.43
	Agarose	Sigma	A6013
	Ethidium bromide	Sigma	E1510
	1 kb ladder	NEB	N3232
	HCl	Supelco	1.43007
	NaOH	Fisher	S318
	Whatman paper	Cytyva	105479223030-6461
	Amersham Hybond XL	GE Healthcare	RPN2020S
	Parafilm	Amcor	PM-996
	Sodium citrate	Fisher	S279
	Hybridization chamber	Techne	Hybridiser HB-1D
	Na2HPO4•2H2O	Fisher	S381
	Na2HPO4•7H2O	Fisher	S373
	Prime-It II Random Primer Labeling Kit	Agilent	300385-52
	dCTP, [α-32P]-3000Ci/mmol 10mCi/ml EasyTide	Revvity	BLU513H100UC
	Ilustra ProbeQuant G-50 Micro Columns	Cytyva	28-9034-08
	Carestream Biomax MS Film	Sigma	Z363006-50EA

Safety warnings

This protocol requires the use of radiolabeled nucleotides and consequently, requires the handling of radioactive materials. Follow your institution's training, regulations and procedures to ensure proper handling of these hazardous materials and ensure researcher’s safety.

Buffers

1 Prepare the following buffers:

1.1 **Lysis buffer:** Tris-Cl pH 8.5 (100mM), EDTA (5mM), NaCl (200 mM), SDS (0.2%) supplemented with proteinase K (100 µg/ml) freshly thawed from frozen stocks.

1.2 **20X SSC buffer:** 3M NaCl, 0.3M sodium citrate.

1.3 **Depurination buffer:** 0.25M HCl.

1.4 **Denaturation buffer:** 0.5M NaOH, 1.5M NaCl.

1.5 **Church buffer:** 1 mM EDTA, 0.5M NaPO4 (pH7.2; critical to check pH), 7% SDS, 1% (w/v) bovine serum albumin (add BSA fresh before usage).

Generation of Southern blot probes

2

Note

While one southern blot probe can be sufficient to determine the genomic integrity of a genome edited locus, we usually recommend a 3’ and 5’ probe in combination with suitable restriction digests to fully evaluate a genome edited genomic locus.

Note

It is advisable to establish robust enzyme digestion and probe functionality on control gDNA before running experiments.

Note

We usually design primers for PCRs with an annealing temperature at 58 C. However, in case of problems to obtain a clean PCR product, the best annealing temperature should be determined specifically for each primer set.

To generate southern blot probes use AccuPrime™ Taq DNA Polymerase, high fidelity; ThermoFisher.12346086 on WIBR3 (parental; RRID:CVCL_9767) gDNA using primers described above.

2.1 Reaction setting: gDNA template (100-200 ng) + 1 µl sense primer (10 µM) + 1 µl anti-sense primer (10 µM) + 5 µl AccuPrime Buffer II + 0.2 µl AccuPrime™ Taq DNA Polymerase, High Fidelity (1U) + molecular grade water (to 50 µl).

2.2 Thermocycler settings: (94 C - 2min) + 30 cycles (94 C - 30 sec + 58 C - 30 sec + 68 C - 1 min/kb) + (68 C - 10 min) + (16 C - ∞).

3 After the reaction is complete, confirm the purity of the probe (PCR product) by running either a small sample or the entirety of it on an agarose gel (0.8%). Purify the gel band or remaining PCR product by using E.Z.N.A.® Gel Extraction Kit by Omega Bio-Tek; Omega Bio-Tek; D5000 (follow manufacturer's instructor on how to prepare each of the necessary reagents).

Cell lysis, gDNA extraction and purification

4

Note

This method to obtain gDNA has been used on human embryonic stem cells on feeder and feeder free conditions and produces samples of sufficient quality for southern blot analyses. It may need to be adjusted/replaced in cases that higher quality gDNA is necessary or other cell types/frozen cell pellets are being used (example: DNeasy Blood & Tissue Kits (69504 QIAGEN).

Remove media from hESC cultures and add Lysis buffer (1 ml/well in 6 well plate and 0.5 ml/well in 12 well plate) and incubate for several hours to Overnight at 37 °C.

5 Transfer up to 750 µL of lysate into a 1.5ml Eppendorf tube and add an equal volume of isopropanol. Vigorously shake the tube (no vortexing to avoid shearing of DNA) until the sample has mixed properly and a white filamentous pellet can be observed inside.

6 Centrifuge your sample on a countertop microcentrifuge (Max speed , ⩾ 13000 x g - 00:05:00 - Room temperature). 5m

7 Decant/aspirate the supernatant and add 1 mL of 70% ETOH.

8 Briefly shake the tube (no vortexing to avoid shearing of DNA) to wash the pellet in 70% ETOH.

9 Centrifuge your sample on a countertop microcentrifuge (Max speed, ⩾ 13000 x g - 00:05:00 - Room temperature). 5m

10 Decant supernatant, aspirate any residual liquid from the tube and leave the cap open to air dry for 00:10:00 - 00:20:00 until pellet starts to become translucent (depending on pellet size time and color may vary). Do not overdry the sample as this could prevent complete resuspension of gDNA. 30m

11 Resuspend the pellet in TE buffer and allow it to slowly solubilize for at least 12:00:00 -24 hours at 56 °C before use. 12h

gDNA digestion

12 Restriction digestion of genomic DNA should be optimized for the enzyme being used.

12.1 Reaction setting: gDNA (10-15 µg) + Restriction Enzyme (4 U per 1 µg DNA, not more than 5 µl enzyme/reaction - check table above to see gene/probe/enzyme combinations used) + 5 ul Buffer (rCutSmart - buffer may vary depending on enzyme being used) + molecular grade water (to 50 µl).

12.2 Incubate samples at 37 °C for 06:00:00 to Overnight incubation (some enzymes may require alternative temperature settings for their efficient performance). 12h

Agarose gel electrophoresis and transfer

13 Digested gDNA should be run on a 0.8% agarose gel with ethidium bromide to separate the sample. A 1 kB ladder should be added next to the samples to be able to discern the molecular weight of bands detected (1/5-1/10 dilution of the marker may be necessary to prevent its signal from overwhelming the blot). Gel size, separation length and

electrophoresis conditions should be optimized for each digest. Electrophoresis voltage should be kept rather low to ensure proper migration and avoid overheating of gel.

14 Once the electrophoresis is complete, carry your gel to a UV reader and collect an image that can be used as reference to locate sample tracks and marker distribution. Trim the areas of the gel above the wells with a razor blade. Make sure to keep track of the wells location. Marks can be made on the edges to be able to confirm the proper orientation.

15 Depurination: in order to reduce DNA fragment size and increase transfer efficiency, gels can be depurinated to create nicks in the strands in the gel. For depurination, incubate gel for 00:15:00 in depurination buffer (0.25M HCl) with gentle shaking - excess shaking may cause the gel to break. 15m

16 Denaturation: incubate the gel in a denaturation buffer for 00:45:00 to 1 hour (0.5M NaOH, 1.5M NaCl). This same solution is used for the transfer. Usual amounts used are ~2L/blot for both denaturation and transfer steps. Prepare more or less as required. 45m

17 DNA transfer to membrane: The DNA transfer is accomplished by the creation of an osmotic potential from one end to the other of the transfer structure. To set up the transfer assembly (see Figure 1 in Green and Sambrook; https://cshprotocols.cshlp.org/content/2021/7/pdb.top100396#cited-by) first prepare a tray (buffer reservoir) and place a glass plate on top in a sideways position. To assemble the transfer assembly, stack the following components from bottom to top on the glass plate:

17.1 Whatman paper sheets are placed on top of the glass so that there is an overhang going into the buffer reservoir tray underneath. Soak the Whatman paper sheets with denaturation buffer.

17.2 Place the gel on top of the Whatman paper in an inverted position so that the upper side where the wells were generated on the agarose gel are on its underside against the paper. This will ensure a flat surface for the DNA transfer increasing its efficiency.

17.3 Place a pre-wetted (denaturation buffer) nylon membrane (Amersham Hybond XL) of similar size to the gel on top of the gel. To prevent bubbles between the gel and the membrane use a serological pipette as a roller to remove any bubbles.

17.4 Place several pre-wetted (denaturation buffer) Whatman paper of similar size to the gel on top of the nylon membrane. Use a serological pipette as a roller to remove any bubbles.

17.5 Place parafilm strips on the edges around the gel on all sides to ensure that no buffer can pass through the gel before reaching the nylon membrane (to prevent buffer shortcuts and increase osmotic potential).

17.6 Place sufficient stack of dry paper towels on top of the transfer assembly and weighted down with a tray and mild pressure (not too much as to damage the gel) to ensure equal transfer through the entire transfer assembly.

17.7 Fill the buffer reservoir tray at the bottom with sufficient denaturation buffer to allow a DNA transfer for 06:00:00 to Overnight . 12h

Membrane blocking and probing

18 Take apart the transfer assembly. Membranes have directionality and it can only be probed from one side, mark the membrane with pencil to keep track of its orientation and wash the membranes with 2xSSC buffer (0.3 NaCl, 0.03M sodium citrate; 3 times 00:10:00). 10m

19 Heat-up the hybridization chamber and cylinders. For each membrane, preheat 2 tubes containing 20 mL of Church buffer (1 mM EDTA, 0.5M NaPO4 (pH7.2; critical to check pH), 7% SDS, 1% (w/v) bovine serum albumin [BSA}, add BSA fresh before usage) to 62 °C - 65 °C .

20 Put the membrane into the cylinders, ensuring the side with the transferred DNA is facing inwards and add in 20 mL of preheated Church buffer . Incubate inside the hybridization chamber for at least 01:00:00 to block the membrane efficiently (constant rotation; 62 °C - 65 °C). 1h

21 In the meantime, prepare the radiolabeled-probes (50 ng DNA/probe) using the Prime-It II Random Primer Labeling Kit (300385-52; Agilent) and dCTP, [α-32P]-3000Ci/mmol 10mCi/ml EasyTide (BLU513H100UC) following the instructions of the vendor.

22 Add the radiolabeled-probe into a resin-based column (Illustra ProbeQuant G-50 Micro Columns) to purify it and add it to the second tube with preheated Church buffer. Remove the blocking solution and add the one containing the radiolabeled probe to the cylinder (Overnight incubation, constant rotation; 62 °C - 65 °C). 1h

Wash and exposure

23 The next steps consist of washes to remove excess and nonspecific bound radiolabeled probes. Washes are done using increasing stringency SSC buffer solutions in the range of 2X (low stringency) to 0.2X (high stringency) + constant 0.2% SDS in all of them. Recommended time is 00:05:00 - 00:10:00 /wash. Use a Geiger counter in between washes to track radioactivity levels. Washes may be done inside the 15m

hybridization chamber canister or inside a sealed container under constant rotation/shaking and 62 °C - 65 °C conditions.

24 Repeat these steps and increase stringency as necessary until the radioactive signal becomes modest. Bands may be detected by the appearance of sudden spikes.

25 Put the membranes into plastic foil and seal the edges to ensure the membranes don't dry.

26 Put it inside a cassette and expose it to a film and leave for 24:00:00 to 72 hours at -80 °C (Exposition time may vary and lengthen), when exposed and seeing the signal adjust time as necessary. The blot will be good to be used for about 2 months, but signal will be lost over time.
If too much background signal is observed, blots can be washed again as previously stipulated and re-exposed to films until the desirable signal is obtained.

1d

Note

Carestream Biomax MS Films are highly sensitive to light exposure. All manipulations before development and fixation need to be performed in a dark room.

Safety information

Wash all materials thoroughly and test the area used for radioactive signals. Clean deeply! Dispose of any one-use materials.

Exposition

27 A film developing system was used to obtain the signal in the film.

Protocol references

https://cshprotocols.cshlp.org/content/2021/7/pdb.top100396#cited-by