Organoid Electroporation using CRISPR RNP method

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

Organoid electroporation using ribonucleoprotein (RNP) CRISPR based approach for highly efficient genome editing.

MATERIALS

Table 1: WENRAFI media composition. Modified from Fujii et al., 2018. Cell Stem Cell.

	A	B	C	D
	Optimised organoid media (replacement of p38i)-WENRAFI	**Stock concentration**	**Volume**	**Final concentration**
	ADF+++	pure	13.08	
	Wnt3a conditioned medium	pure	25 ml	50%
	R-spo conditioned medium	pure	10 ml	20%
	Primocin (Invivogen #ant-pm-1)	50mg/ml (500x)	100µl	500 µg/mL
	B-27® Supplement (Invitrogen #17504-044)	50x	1000 µl	1x
	Nicotinamide (Sigma #N0636, in water)	1 M (100x)	500 µl	10 mM
	N-Acetylcysteine (Sigma # A9165, in water)	500 mM (400x)	125 µl	1.25 mM
	A3801 (Tocris #2939,in DMSO,)	5 mM (10,000x)	5 µl	500 nM

	A	B	C	D
	mEGF (Invitrogen Biosource #PMG8043)	100 ng/µl (2,000x)	25 µl	50 ng/mL
	mNoggin (Peprotech #250-38)	100 ng/µl (1,000x)	50 µl	100 ng/mL
	IGF-1 (Biolegend, 590904)	100 ng/µl	50 µl	100 ng/mL
	FGF-2 (Peprotech, #100-18B)	100 ng/µl	25 µl	50 ng/mL
	Total		**50 mL**	

Table 2: ENAFI media composition

	A	B	C	D	E
	ENAFI (EGF, Noggin, ADF, FGF2, IGF1)	**Stock concentration**	**Final concentration**	**ENAFI+ Y+Chir (48h before)**	**ENAFI+ Y+Chir+DMSO (24h before and elec day)**
	ADF+++	pure		24010	23697.5
	Primocin (Invivogen #ant-pm-1)	50mg/ml (500x)	500 µg/mL	50	50
	B-27® Supplement (Invitrogen #17504-044)	50x	1x	500	500
	Nicotinamide (Sigma #N0636, in water)	1 M (100x)	10 mM	250	250
	N-Acetylcysteine (Sigma # A9165, in water)	500 mM (400x)	1.25 mM	62.5	62.5
	A3801 (Tocris #2939,in DMSO,)	5 mM (10,000x)	500 nM	2.5	2.5
	mEGF (Invitrogen Biosource #PMG8043)	100 ng/µl (2,000x)	50 ng/mL	12.5	12.5
	mNoggin (Peprotech #250-38)	100 ng/µl (1,000x)	100 ng/mL	25	25

	A	B	C	D	E
	IGF-1 (Biolegend, 590904)	100 ng/µl	100 ng/mL	25	25
	FGF-2 (Peprotech, #100-18B)	100 ng/µl	50 ng/mL	12.5	12.5
	Y-27632	10 mM	10 uM	25	25
	CHIR99021	10 mM	5 uM	25	25
	DMSO		1.25%		312.5
	Total			25mL	25mL

Organoid Expansion (Day -5)

3h

1 Expand organoids as previously described. Aim for 10-20 wells of organoids for sufficient cell numbers, depending on the numbers of conditions you want to test. Feed organoids with WENRAFI media (Table 1).

Media Preparation (Day -2)

10m

2 48 h before electroporation, replace the medium with 250 µl of ENAFI medium supplemented with 5 µM CHIR99021 and 10 µM Y-27632 (Table 2).

Media Preparation (Day -1)

10m

3 24 h before electroporation, replace the medium with 250 µl of ENAFI medium supplemented with 5 µM CHIR99021, 10 µM Y-27632 and 1.25% (vol/vol) DMSO (Table 2).

Single Cell Dissociation (Day 0)

1h

4 Remove the medium from the organoids and add 500 µL of TrypLE Express supplemented with 10 µM Y-27632 to each well. Scrape the Matrigel off the bottom of the wells with a 1,000-µl pipette. Split the organoids in 2-4 15 mL Falcons to have smaller volume for the dissociation process.

5 Place the tubes in a 37 °C water bath for 00:30:00. Pipette vigorously every 5 min, 10 times with 10 mL pipette and 10 times with a 1,000-µl pipette with broken tip. 30m

6 Thaw Cas9 and guide On ice.

7 Add basal medium up to 10 ml and centrifuge for 4 minutes at 500g. Combine separate Falcon tubes at this stage to have a bigger pellet.

8 Aspirate and discard the supernatant. If pellet is loose, do a second centrifugation step in an ependorf with 500-1000 µl of media left.

9 Aspirate and discard supernatant. Add 500 µL of Opti-MEM media and pipette well to mix.

10 Count number of cells with a haemocytometer (take 10 µl). Determine number of conditions (100,000 cells per condition). You will need to include negative control, no Cas9.

Making RNP complex

30m

11 Mix 1 µL of Cas9 and 1 µL of guide (1:3.33 ratio), you will need to add 2 µl per condition. 20m

Standard concentration: 5ug True Cut Cas9 v2 (Invitrogen, A36499- 500 µg at 5µg/µl) and 100pmol synthetic guide (Synthego- custom made, supplied 3 nmol lyophilised reconstituted with 30 µl water for 100pmol/µl).

Make complex and leave 00:20:00 at Room temperature.

12 Spin and pellet correct number of cells before washing with 300 µL of PBS. Centrifuge at 500g for 4 minutes.

13 While washing with PBS make P3 suppl buffer (20 µl/reaction) (Lonza, V4XP-3032).

Buffer P3: 16.4 µl and Supplement 1: 3.6 µl for a total of 20 µl per condition (recommended to make at least 10% excess for pipetting error). Supplemented with 10µM Y-27632, leave at Room temperature.

14 Completely remove and discard the supernatant. Resuspend in 20 µL of P3 buffer supplemented with 10 µM Y-27632 per condition.

15 Add 2 µL of RNP complex per condition.

16 Mix well and load 20 µL into electroporation chamber (16-well nucleovette strips).

Electroporation 30m

17 Leave 00:10:00 at Room temperature before electroporation. 10m

18 Perform electroporation on Lonza Amaxa 4D Nucleofector with program DS-138.

19 Incubate at 37 °C for 00:10:00 . 10m

Seeding cells 20m

20 Add 80 µL of warm ENAFI media+ Y+ Chir+DMSO to each chamber. Remove 100 µl into seperate ependorfs. Wash each chamber with another 100 µL of media to ensure you have taken all cells.

21 Centrifuge at 500g for 4 minutes.

22 Remove and discard the supernatant and suspend the pellet with 20-25 µl Matrigel per well. Set up 2 wells per condition.

23 Place the plate in a 37 °C incubator for 00:10:00 to solidify the Matrigel. 10m

24 Once matrigel has solidified, add 250 µl of ENAFI medium supplemented with 5 µM CHIR99021, 10 µM Y-27632 and 1.25% (vol/vol) DMSO (Table 2) to each well.

Media change (Day +1) 10m

25 Next Day: change media back to WENRAFI (Wnt and Rspo conditioned, Table 1) supplemented with 10 µM of Y-27632.

DNA extraction and screening (Day +7)

5h

26 7 days after electroporation, extract DNA from half or a third of the well using PicoPure DNA extraction kit (Invitrogen, KIT0103). Perform 65 °C lysis step for 03:00:00 hours. 3h

27 Perform PCR using primers that span the guide (500-800 bp) and submit for Sanger sequencing in both directions.

28 Analyse sanger trace using ICE Synthego. You will need to upload a control trace (No Cas9) for each edited trace.
https://ice.synthego.com/#/