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

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Enzymatic padlock probe preparation

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ABSTRACT

This protocol accompanies the manuscript Mapping Human Tissues with Highly Multiplexed RNA in situ Hybridization (https://doi.org/10.1101/2023.08.16.553610). It protocol outlines the steps for the enzymatic amplification and preparation of padlock probes from an oligo pool. This strategy has a lower upfront cost compared to individual synthesis of probes and thus permits the use of tens of thousands of padlock probes per probe set. Additionally, the oligo pool serves as an unlimited source of padlock probes, as it can be expanded by PCR amplification upon need.

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MATERIALS

Reagents

	Material	Supplier	Catalog number
	KAPA SYBR FAST qPCR kit	Roche	KK4602
	Glycoblue Coprecipitant (15mg/mL)	ThermoFisher Scientific	AM9515
	Sodium Acetate (3M), pH 5.5, RNase-free	Invitrogen	AM9740
	Lambda Exonuclease 1,000U	NEB	M0262S
	USER	NEB	M5505L
	DpnII	NEB	R0543L
	Novex TBE-Urea Sample Buffer (2X)	ThermoFisher Scientific	LC6876
	SYBR Gold	Invitrogen	S11494
	Nanosep Columns; 0.2 um filter	VWR	29300-646
	QIAquick PCR purification kit	Qiagen	28104
	ssDNA/RNA clean & concentrator kit	Zymo Research	D7011
	Ammonium persulfate	Sigma-Aldrich	A3678
	TEMED	Fisher Scientific	17919
	40% Acrylamide/ Bis solution (29:1)	Fisher Scientific	BP1408-1

Primers

	Primer name	Vendor	Sequence	Usage
	pAP1V41U	IDT	G*T*AGACTGGAAGAGCACT GTU	Amplification of kidney probe set
	AP2V4	IDT	/5Phos/TAGCCTCATGCGTA TCCGAT	Amplification of kidney probe set
	AP1V7U	IDT	A*A*GCAAGATTCTCGTCGA G/3deoxyU/	Amplification of brain probe set
	AP2V7	IDT	/5Phos/TGTAAGGCACATCT CGGATC	Amplification of brain probe set
	RE_DpnII_V7 N	IDT	GCACATCTCGGATCNNNN	Amplification of brain probe set

BEFORE START INSTRUCTIONS

The main starting material is an oligo pool with probe-set specific amplification primers on the 5' and 3' ends. In the example below, AP1V7 and AP2V7 are the primer pair that specifically amplify our probe set of interest.

oligo pool pre-amplification

1 Resuspend the oligo pool in water to a concentration of 7.02 ng/uL. Dilute the pool 100 times and PCR to amplify the brain probe set. 4m 30s

	reagents	1x volume (ul)
	100x diluted oligo pool	5
	10uM AP1V7U primer	2
	10uM AP2V7 primer	2
	2x KAPA SYBR FAST qPCR master mix	25
	ultrapure water	16

PCR program:
95 °C , 00:00:30 -> (95 °C , 00:00:30 -> 55 °C 00:00:45 -> 72 °C 00:00:45)x17 cycles -> 72 °C , 00:02:00 -> 15 °C , hold

2 Purify the PCR product by QIAquick PCR purification kit and elute each QIAquick column with 50 µL ultrapure water

Use Qubit with HS dsDNA kit to quantify the concentration and dilute the PCR product to 10nM by adding ultrapure water. This is called "10nM oligo pool 1st amplicon" in the following section.

PCR mass production 50m

3 Prepare PCR production mix in a 15mL tube and add load 100 µL to each well of a 96-well plate 5m

	reagents	1x volume (ul)	100x volume (ul)
	10nM oligo pool 1st amplicon	0.2	20
	2x KAPA SYBR FAST qPCR master mix	50	5000
	100uM AP1V7U primer	0.4	40
	100uM AP2V7 primer	0.4	40
	ultrapure water	49	4900

run PCR program to amplify the oligo pool
98 °C , 00:01:00 -> (98 °C , 00:00:30 -> 55 °C 00:00:45 -> 72 °C 00:00:45)x14 cycles -> 72 °C , 00:02:00 -> 4 °C , hold

4 Pool the PCR production mix from the 96-well plate and prepare the ethanol precipitation mix in 5r 1h 30m tubes. (One 96-well plate can split into 8 5mL-tubes)

	reagents	1x volume
	PCR product	1200
	100% ethanol	3000
	glycoblue coprecipitant	4
	3M NaOAc	120

Mix the tubes by shaking and incubate them in a -80 °C freezer for 01:00:00 .
Centrifuge the tubes at 4,000rpm, 4 °C for 00:30:00 and discard the supernatant

5 Add 800 µL 80% ethanol to each 5mL tube and transfer the solution into another 1.5mL tube. (8 5m 5mL-tubes transfer into 8 1.5mL tubes).
Centrifuge at 14,000rpm, 4 °C for 00:05:00 and discard the supernatant.

6 Air-dry the DNA pallets on ice bucket in AirClean hood for 00:10:00 10m
Rehydrate DNA pallets with 50 µL ultrapure water
Pool the DNA solutions and use 8 QIAquick columns to purify.
Use Nanodrop to quantify the concentration.
Aliquot 3uL for TBU gel check

Lambda Exo digestion 2h

7 Prepare Lambda Exo digestion mix and split it into PCR tubes (100 µL per tube). Adjust the volum 2h of DNA amplicon and ultrapure water so that the DNA amplicon per tube is around 5 µg

	reagents	1x volume (ul)	16x volume (ul)
	DNA amplicon	25	400
	10x Lambda Exo buffer	10	160
	Lambda Exo (5U/uL)	10	160
	ultrapure water	55	880

Run PCR program: 37 °C , 02:00:00 -> 4 °C ,hold

8 Use 16 ssDNA/RNA Zymo-spin columns to purify the Lambda Exo-digested products and elute the columns with 50 µL ultrapure water
Use Nanodrop to quantify the concentration.
Aliquot 3uL for TBU gel check

USER digestion

3h

9 Prepare USER digestion mix and split into PCR tubes (80 µL per tube). Adjust the volume of ssDN 3h and ultrapure water so that the ssDNA per tube is less than 2 µg .

	reagents	1x volume (ul)	12x volume (ul)
	ssDNA	66.7	800
	USER (1U/uL)	5	60
	10x DpnII buffer	8	96
	ultrapure water	0.3	4

Run PCR program: 37 °C , 03:00:00 -> 4 °C ,hold.
Aliquot 3uL for TBU gel check

DpnII digestion

5h 5m

10 Prepare DpnII oligo mix and add 15 µL to each PCR tube 5m

	reagents	1x volume (ul)	12x volume (ul)
	10x DpnII buffer	2	24
	100uM RE_DpnII_V7N primer	5	60
	ultrapure water	8	96

Run PCR program: 94 °C , 00:02:00 -> 37 °C , 00:03:00 -> 4 °C , hold

11 Add 5 µL DpnII enzyme (10U/uL) to each PCR tube (total volume is 100 µL per tube) and mix 5h pipetting.

Run PCR program: 37 °C , 05:00:00 -> 4 °C , hold

12 Use ssDNA/RNA Zymo-spin columns to purify each PCR tube and elute each column with

50 µL ultrapure water

Use Nanodrop to quantify the concentration

Aliquot 3uL for TBU gel check

TBU gel check (optional)

30m

13 Pre-run one 12-well TBU-gel for at least 00:10:00

40m

Prepare TBU gel check samples

	sample	input (ul)	ultrapure water (ul)	2x Novex buffer (ul)
	10 bp DNA ladder	1	4	5
	1: PCR product	1	4	5
	2: Lambda Exo	1	4	5
	3: Lambda Exo +USER	1	4	5
	4: Lambda Exo +USER +DpnII	1	4	5

Notes: If PCR product and Lambda Exo sample look overloaded, dilute the sample by 10-20 times before TBU gel check.

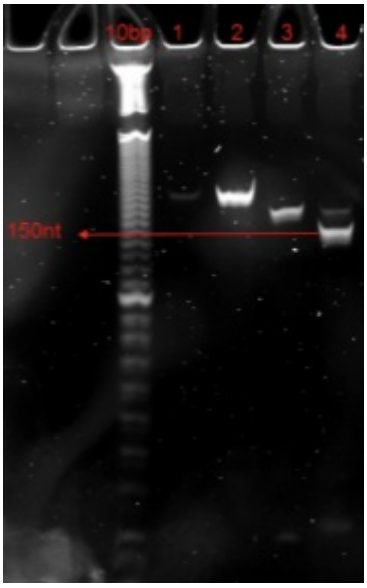
Incubate TBU gel check samples at 70 °C for 00:05:00 and immediately put on ice for 00:05:00

Flush each well with TBE running buffer to remove excessive urea in the 12-well gel

Load the TBU gel check samples to the 12-well TBU gel

Run gel electrophoresis at 220-240 V for 00:20:00

Example TBU gel for a padlock probe set with the final size of 150nt:



Size selection

2h 51m

14 Pre-run 1-well TBU gels for at least 00:10:00 — 20m

Prepare enzyme-digested probes: add 180 µL 2x Novex buffer to 180 µL enzyme-digested probes

Incubate the samples at 70 °C for 00:05:00 and immediately put on ice for 00:05:00

Flush each well with TBE buffer to remove excessive urea in each well.

15 Load samples to 1-well TBU gel. — 20m

Notes: Cap the amount of enzyme-digested probes per 1-well TBU gel at 2 µg to avoid overloading and add the equivalent volume of 2x Novex buffer. The rest of the enzyme-digested probes can be stored at -20 °C freezer for the next round of size selection.

Run gel electrophoresis at 220-240 V for 00:20:00

16 Cut enzyme-digested probes at the right size and transfer the cut-out gels to 0.5mL tubes (with holes the bottom) on top of 2mL tubes — 5m

Centrifuge the tubes at 15,000rpm for 00:05:00 to shred gels

Transfer the remaining gel in the 0.5mL tubes to the 2 mL tubes

17 Add 450 µL 1x TE buffer, pH 8.0 to each 2mL tubes and mix the tubes by shaking 2h

Freeze and thaw the solution in the -80 °C freezer, Overnight and vortex the tubes in a 37 °C incubator for 02:00:00

18 Centrifuge the tubes at 15,000rpm, Room temperature for 00:03:00 6m

Transfer the clear supernatant to Nanosep columns and centrifuge at 15,000 rpm, Room temperature for 00:03:00

Repeat Nanosep filtering once and transfer the flow-through to 1.5 mL tubes

Ethanol precipitation 1h 45m

19 Add 1 mL 100% ethanol , 1.4 µL glycoblue coprecipitant , 45 µL 3M NaOAc, pH 5.2 to each tube and mix by shaking

20 Precipitate probes in a -80 °C freezer for at least 01:00:00 1h 30m

Centrifuge at 10,000 rpm, 4 °C for 00:30:00 and discard the supernatant

21 Wash the pallets with 750 µL cold 80% ethanol and centrifuge at 14,000rpm at 4 °C for 00:05:00 5m

22 Discard the supernatant and air-dry the pallets in AirClean hood for 00:10:00 10m

23 Rehydrate the pallets with 10 µL ultrapure water per tube and pool the probe solution together

Use Qubit with ssDNA kit to quantify the concentration of probe solution