**PAS-Seq protocol**

**RNA fragmentation (starting material consists of 5µg of total RNA resuspended in 10µl of water)**

- Add 10µl of 5X Superscript RT buffer (the one from Life technologies).

- Incubate at 94C degrees for 5 minutes and 30 seconds.

- Immediately place on ice for 5 minutes.

**Reverse-transcription:**

- To the fragmented RNA add 2.5µl of dNTPs (10mM stock).

- 2.5µl of 100mM DTT.

- 2µl of RT primer (anchored oligo(dT), see below for sequences).

- 22µl of water.

- 1µl of Superscript III

Incubate as follow:

- 25˚C for 5 minutes.

- 50˚C for 50 minutes.

- 85˚C for 5 minutes.

- Hold at 4˚C for 5 minutes.

- After the reaction is over, add 100 units of RNaseI (Life Technologies AM2294) and incubate at RT for 10 minutes.

**Size selection of cDNAs products:**

- To the RT samples, add 20µl of 3X denaturing loading buffer (see recipe at the end).

- Heat denature the RT products at 65˚C for 5 minutes.

- Run the heat denatured RT products on a 8M urea, 10% acrylamide (19:1), 0.5X TBE gel at 35watts for 1hour and 45minutes.

- Stain the gel with Sybr gold (Life technologies S-11494).

- Cut the cDNA products that run around 160-200nt.

- Crush the gel piece and add 800µl of elution buffer (300mM NaCl, 10mM EDTA).

- Nutate overnight at RT.

- Recover the supernatant and pass through a Spin-X column (Corning CLS8162-24EA) and spin at 10000xg for 3 minutes.

- Recover the flow-through, add 20µg of Glycogen and 1 volume of Isopropanol.

- Incubate at -20˚C for 30 minutes and spin at Vmax for 45 minutes to pellet the DNA.

- Wash the pellet in 800µl of 70% ethanol and spin at Vmax for 5 minutes.

- Air dry the pellet and resuspend it in 11µl of water.

**Circularization of cDNAs**

To the 11µl cDNA add the following:

- 2µl of 10X CircligaseI Reaction buffer.

- 1µl of 1mM ATP.

- 1µl of 50mM MnCl2.

- 4µl of 5M betaine (Sigma B0300-1VL).

- 1µl of Circular ligase (Epicentre CL4115K).

Incubate at 60˚C for 4 hours and then at 85˚C for 10 minutes to inactivate the enzyme.

**PCR amplification**

Using the proofreading polymerase of your choice take 2 to 4µl of the circularized product (for a 30µl final PCR reaction volume) using Illumina’s PE1.0 and 2.0 primers and amplify it for 10-14 cycles (you have to test different number of cycles to use the one that gives you the best yield).

**Sequence of RT-primers (the red part corresponds to the barcode):**

|  |
| --- |
| /5Phos/GGNNNNNATCACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNCGATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNTAGCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNGCTCCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNACAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNCAGATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNTCCCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNGGCTAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNAGTCAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNCTTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNTGAATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |
| /5Phos/GGNNNNNGTAGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTVN-3` |

**2X loading buffer recipe:**

2 mL 5X TBE, 1.2 g Ficoll Type 400, 4.2 g Urea, 2 mg bromophenol blue, 2 mg xylene cyanol,

up to 10 ml ddH20; store at 4°C

- heat to get into solution or nutate O/N

- add dyes after adjusting the volume to 10 mL