Electronic Supplementary Material (ESI) for Lab on a Chip.

Sequencing of Human Genomes Extracted from Single Cancer Cells Isolated In a Valveless Microfluidic Device

Supporting Information

Diffusion during amplification step

Here we examine whether diffusion from the outlets through the channel contributes to a significant loss of material from the outlet during the overnight amplification (Figure S10).

Then we examine the diffusion into the outlet channel, through the trap and towards the feeding channel (Figure S10A). We need to solve the diffusion equation in one dimension of the sample (fragments of single stranded DNA), the enzyme (Φ 29 polymerase, 66kDa) and the amplicons of the amplification reactions (single stranded DNA, a few kb in length).

First we estimate diffusion coefficients. The sample DNA, product of the cell lysis, is single stranded however we do not know the length of the fragment.

We do know that by the time the amplification starts, the sample DNA and the amplicons are single stranded. The persistence length of ssDNA is about 1nm [1] at the ionic strength in the range of 0.1-1M. ssDNA amplicons have a length in the range of a few kb. For 1kb, the corresponding radius of gyration is 18nm [1] and the diffusion constant calculated from the Stokes-Einstein relation is 12 $\mu m^2.s^{-1}$.

The denaturation solution is basically a solution of KOH, so we use the diffusion coefficient of small ions in solution D = 2000 $\mu m^2.s^{-1}$. [2]

The Φ29 enzyme has a molecular weight Mw of 66kDa. We estimate the radius using [3]:

$$R_{min} = 0.066\, nm\, \frac{{M_w}^{1/3}}{Da} \quad . \qquad (1)$$

Here R_{min} is, in nanometers, the minimum radius of a protein of molecular weight M_w, in Daltons, assuming a spherical form. This is the upper bound estimate of the diffusion coefficient. We find R_{min} = 2.67 nm for Φ29. We calculate the diffusion coefficient:

$$D = \frac{k_B T}{6\pi\eta R_{min}} \, . \qquad (2)$$

and find D=81 $\mu m^2.s^{-1}$ for Φ29 in water.

We want to find out if the diffusion through the channels contributes significantly to the loss of material from the outlets during amplification overnight. We then solve the 1-dimension diffusion across the

channel leading to the outlet. The channel has a depth of 30 µm, a width of 100 µm. The length varies from outlet to outlet but we can calculate an estimate assuming a length of ~ 1cm.

As a first estimate of how much material would be lost from the outlet well through diffusion, we calculate how much solution is contained in the outlet channel (30nL, Figure S10B). This internal volume is negligible compare to the solution contained in the outlet well (in the order of 10µL, three orders of magnitude larger) where the amplification is performed. This tells us that the amount of DNA or enzyme that is contained in the outlet channel, should it be totally filled with solution from the outlet, is minute compare to the amount of DNA and enzyme contains in the reaction mix in the outlet

To refine this estimate, we can calculate the flux of materials escaping the outlet through such a channel. Here we can assume that a sink is placed at the other extremity of the channel (clearly a worst case scenario). At steady state:

$$\frac{dC}{dt} = 0 = D\frac{d^2C}{dx^2} \quad . \tag{3}$$

The standard solution is:

$$C(x) = 0 = k_1 x + k_2 \quad . \tag{4}$$

We use the following boundary conditions: first, the concentration in the outlet is constant so that C(0, t) = C_0. Then we say that the feeding channel, at the end of the outlet channel, works as a sink so that C(L, t) = 0. We write that the gradient in the outlet microchannel is constant with a slope

$$C(x) = -C_0(\frac{x}{L} - 1). \tag{5}$$

The loss of material is the flux from the outlet through the microchannel is

$$J = -D\ \frac{dC}{dx} = \ C_0 \frac{D}{L}. \tag{6}$$

The microchannel has a cross-sectional area A = h w = 3000 µm^2, we can calculate the flux in mol.s^{-1} through the channel (Table S3). We conclude that the loss of material from the outlet through the channel is negligible.

Table S3: Estimated loss of DNA, enzyme and small ions from the outlet through the channel assuming 10 µL of mix in the outlet containing 6 pg of ssDNA sample as 1kb fragments and 1 mg/mL of Φ29.

Entity	**Typical Conc.**	**D in (µm)^2.s^{-1}**	**Flux *JA***	**Total loss for 12h**
ss-DNA	0.6 ng/mL	12	2 10^{-12} ng.s^{-1}	10^{-4} pg

Φ29	1 mg/mL	81	24 10^{-12} mg.s^{-1}	1 ng
Small ions	1 M	2000	0.6 10^{-12} mole.s^{-1}	26 nmole

References:

[1] Sim, A. Y. L.; Lipfert, J.; Herschlag, D.; Doniach, S. 'Salt dependence of the radius of gyration and flexibility of single-stranded DNA in solution probed by small-angle x-ray scattering.' Physical Review E 2012, 86, 021901.

[2] Bruus, H. 'Theoretical microfluidics'; Oxford University Press, 2007; Vol. 18; p 346

[3] Erickson, H. P. 'Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy.' Biological Procedures Online 2009, 11, 32–51.

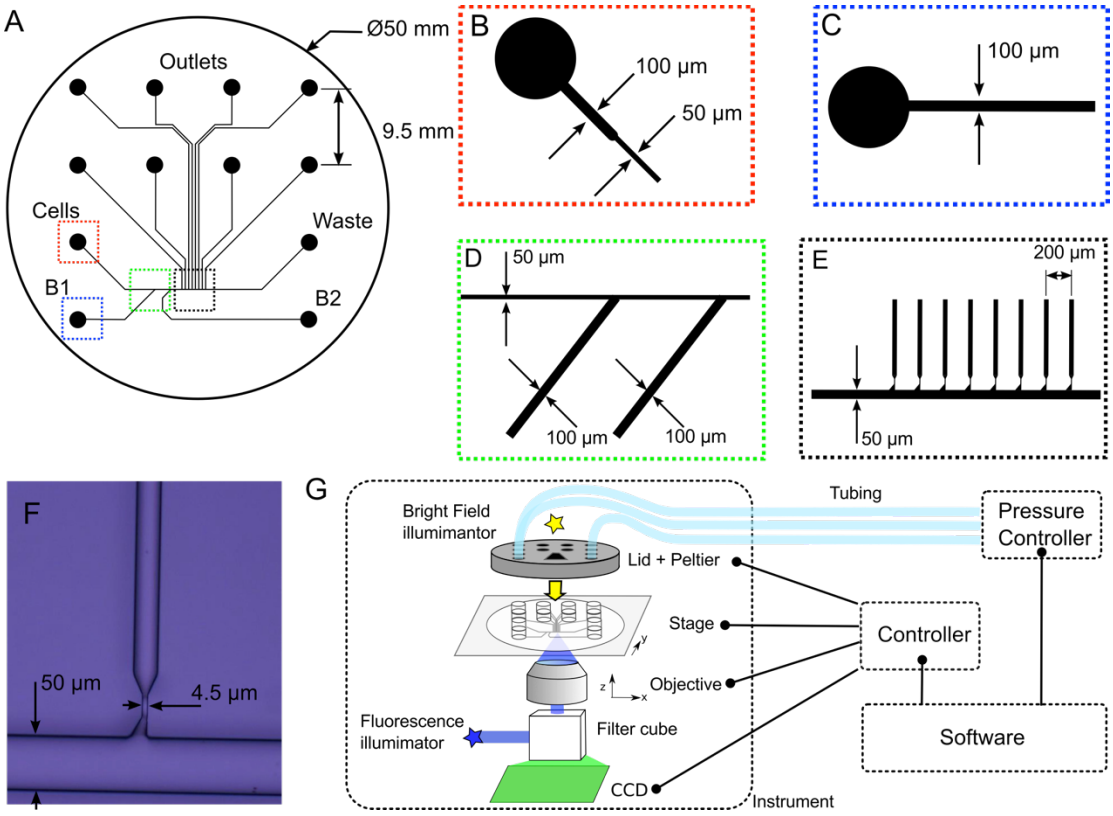

Figure S1. Dimensions of the microfluidic chip for cell trapping and lysis. (A) overall design with 12 LUER connectors. The structure is a one-layered design with 30 µm deep channels. (B) Cell inlet is a 100 µm-wide channel then becomes 50 µm-wide. (C) The channels from inlets B1 and B2 are 100 µm-wide. D) schematics of the connection between the feeding channel connecting the cell inlet to the waste and the two buffer inlets from B1 and B2. (E) Eight traps are placed at a 200 µm spacing (F) Image of the cell trap on the Nickel shim (G) Schematics of the set-up.

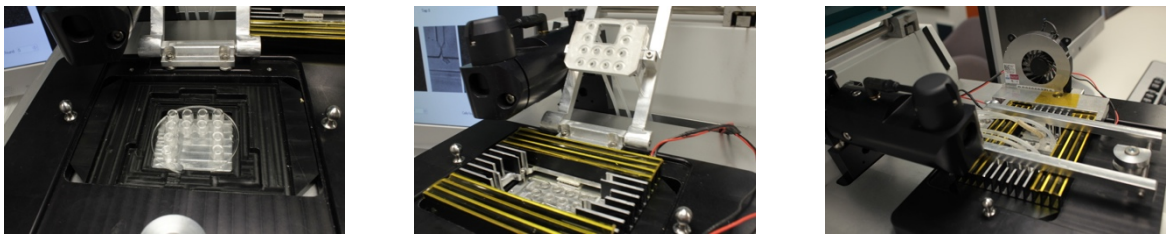

Figure S2. Photographic images depicting the chip, thermo block and clamp respectively.

Protocol S3. Protocol for DNA extraction and amplification from single cells using a microfluidic chip and a custom build 'Cell-O-Matic' instrument

NOTE: We refer to instructions in the Cell-O-Matic instrument software.

NOTE: all buffers/solutions used in experiment must be sterile

Preparation of cells:

1. Trypsinize
2. Wash out medium/trypsin
3. Filter cells 0.2 µm
4. Suspend 500 000 cells (no more!) in 500 µL degassed FACSFlow+0.1mg/mL BSA
5. Add 1 µL of calcein-AM
6. Incubate at 37 °C for 30 minutes
7. Switch on in the following order:

   Computer

   Router

   Cell-O-Matic instrument

   Temperature controller

   Fluigent pressure controller, MFSC-EZ. NOTE: the value of the Input pressure on the screen should be approx 800, if not, correct it with a valve according to manufacturer's instructions.

. Run Cell-O-Matic instrument software

   In pop up window choose: router – CelloMaticMU2 and click 'Connect'

Chip preparation

1. Degas and filter FACSFlow + 0.1% Triton X-100 and FACSFlow (filtered) + 0.1 mg/ml BSA
2. Prepare (=wet) chip
3. Add 40 µL degassed FACSFlow+ 0.1% Triton X-100 to inlet #3
4. Add 80 µL degassed FACSFlow+ 0.1mg/mL BSA to inlet #2

Load chip

1. Open green cover
2. Lift holder and thermo block, see Figure S2 for photographic images
3. Place chip at the bottom of thermo block
4. Mount thermo block with a chip ensuring wires are not pressed
5. Put holder back and tightly lock

6. Close green cover

7. In Cell-O-Matic instrument software, 'Live View' tab, click 'Calibrate positions'.

8. Camera settings: Bright Field

9. Motor settings → Presets: Trap 1 and search for trap 1 by changing position values (horizontal and vertical and focus; red crosshair should be above triangular trap)

10. Click 'Save Current Position' under Trap1 section

11. Click 'Go to Trap 8' and set red crosshair on the channel just above triangular trap

12. Click 'Save Current Position' under Trap 8 section then OK at the bottom to save

13. Pressure control panel: click 'Zero' on all channels to calibrate pressures.

14. Set pressure on Channel #3: 200 (mbar), click Apply and observe Inlet #4 for a few seconds (to be able see anything: change preset to Inlet #4, zoom out, open cover and use external source of light e.g. torch in smartphone)

15. Stop (change value to 0 and click Apply) as soon as you notice some fluid in Inlet #4

NOTE: if you run too long you will introduce air bubbles into system which are almost impossible to remove = prepare new chip

16. Set pressure on Channel #2: 200 (mbar), click Apply and observe Outlets #1-8 for a few seconds (to be able see anything: change preset to Outlet #1, zoom out, open cover and use external source of light e.g. torch in smartphone, then repeat for other outlets)

17. Stop (change value to 0 and click Apply) as soon as you notice some fluid in all outlets

NOTE: if you run too long you will introduce air bubbles into system which are almost impossible to remove = prepare new chip

18. From presets choose 'Load'

<u>Cell capture</u>

1. Remove chip from the instrument

2. In a cell culture hood: wash 3x all inlets and outlets with 50 µL sterile PBS

3. To Inlet #1-3 add 40 µL of FACSFlow+0.1mg/mL BSA

4. To Inlet #1 add 10 µL of stained cell suspension (on the top of FACSFlow + BSA, do not mix)

5. Place chip in the instrument

6. Go to Work Flow tab

7. Click 'New Run...', give a name for your experiment

8. Click 'Calibration Pressure', then 'Calibrate Positions', finally 'Scan Traps 1'

9. Ensure 'Apply Immediately' is not chosen

10. Set pressures: Channel #1: 5 mbar, Channel #2 and #3: 4 mbar, click 'Apply'

11. Observe process, stop (change value on Channel1 to 0 and click Apply) when 7-8 traps are occupied by viable (fluorescent cells)

NOTE: if there are any problems e.g. no cells in feeding channel you can check in Live View tab different locations on chip to see where are the cells, are there any air bubbles blocking etcetera.

12. Run pressure 50 mbar on Channel#2 and #3 to ensure that unwanted cells are removed from feeding channel

13. Click 'Check passed' to close window, then 'Load' to remove chip from the instrument.

First lysis step:

1. In a cell culture hood: first remove cells from Inlet1, wash inlets #1-3 thoroughly

2. Add 8 µL of PCR water in all outlets just next to channel entrance

3. Add 20 µL of lysis buffer I (0.5x TBE+0.5% Triton X-100 v/v) to inlet#2

4. Insert chip in the instrument, click OK in a pop up window 'Please insert chip'

5. Go to 'Scan traps1', apply pressure of 10 (mbar) on Channel#2 for 15 min (membrane lysis, cytosol with RNA will be moved to outlets, nucleus will stay in a trap)

6. Stop by zeroing pressure, click 'Check passed', then 'Load', remove chip

7. In a cell culture hood: transfer PCR water containing RNA from outlets to PCR tubes and freeze in -20°C immediately

Second lysis step:

1. In a cell culture hood: remove lysis buffer I, wash once with PBS and add 20 µL of D2 Buffer (Qiagen REPLI-g UltraFast Midi Kit)

2. Insert chip in the instrument, click OK in a pop up window 'Please insert chip'

3. Go to 'Scan traps1', apply pressure of 10 mbar on Channel#2 for 15 min (alkaline nucleus lysis)

4. Stop by zeroing pressure, click 'Check passed', then 'Load', remove chip

5. In a cell culture hood: on top of D2 Buffer add 20 µL of Neutralization Buffer (Qiagen REPLI-g UltraFast Midi Kit)

6. Insert chip in the instrument, click OK in a pop up window 'Please insert chip'

7. Go to 'Scan traps1', apply pressure of 10 mbar on Channel#2 for 15 min (neutralization; all gDNA should be in outlets)

On chip whole genome amplification (WGA):

1. Prepare WGA reaction mix (Qiagen REPLI-g UltraFast Midi Kit):

   per sample: 2.25 µL H_2O

   7.25 µL reaction buffer

   0.5 µL polymerase phi29

2. Stop by zeroing pressure, click 'Check passed', then File → Quit, remove chip

3. In a cell culture hood: add 10 µL of WGA reaction mix to all outlets

4. Insert chip in the instrument, run software SC Interface from desktop

5. Click 'Open', choose and open file 'Peltier-setup 09-09-2015.sc'

6. 'Regulator' → 'Connect', then SEND button on the top right to start WGA (overnight)

7. On the next day, remove chip from the instrument, collect amplified gDNA into PCR tubes (ready for sequencing)

8. Prepare amplified cDNA following protocol Qiagen REPLI-g WTA Single Cell Kit (all steps can be run in PCR machine, look for them in folder WTA)

OPTIONAL

Multiplex chromosome check (to see efficiency of WGA)

NOTE: include negative (no DNA) and positive (bulk DNA extracted from LS174T)

1. Thaw on ice LS174T DNA, dH_2O, RedTaq polymerase, MgCl2, multiplex primers

2. Prepare MasterMix (9 µL x [n samples +1]):

    5 µL RedTaq

    0.6 µL MgCl2 (25 mM)

    2 µL multiplex primers (10 uM)

    1.4 µL dH2O

3. Transfer ~10 µL of amplified DNA from outlets to PCR tubes, mix and transfer 1 µL of DNA to new PCR tubes

4. Add 9 µL of MasterMix to PCR tubes and mix

5. Put lids on PCR tubes

6. Place tubes in PCR machine and run from folder WGA 'MULTIPLEX CHROMOSOME CHECK' program (~2.5 hrs long):

    - Denaturation 94°C for 2 min

    - 50 cycles of: - Denaturation 94 °C for 1 min

        - Annealing 60 °C for 1 min

        - Elongation 72 °C for 1 min

    - Elongation 72 °C for 7 min

    - 4°C hold

Gel electrophoresis

1. Prepare 4% agarose solution (electrophoresis grade), 2g in 50 ml of 1xTBE

2. Heat in microwave for 2 x 50 sec, 750 W

3. Let agarose solution cool down for 5 min in RT, add 5 µL SYBR Safe (1:10 000)

4. Prepare gel tray using tape

5. Pour the agarose into a gel tray, place well comb (avoid air bubbles!)

6. Wait approx. 30 min until gel has completely solidified

7. Once solidified, remove tape and comb, and place the agarose gel into the electrophoresis unit

8. Fill the unit with 1x TBE until the gel is covered (no air bubbles in the wells!)

9. Load 5 µL of sample

10. Run the gel at 100V for approx. 1.0h (or 120V for shorter time if needed)

11. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box

14. Visualize DNA

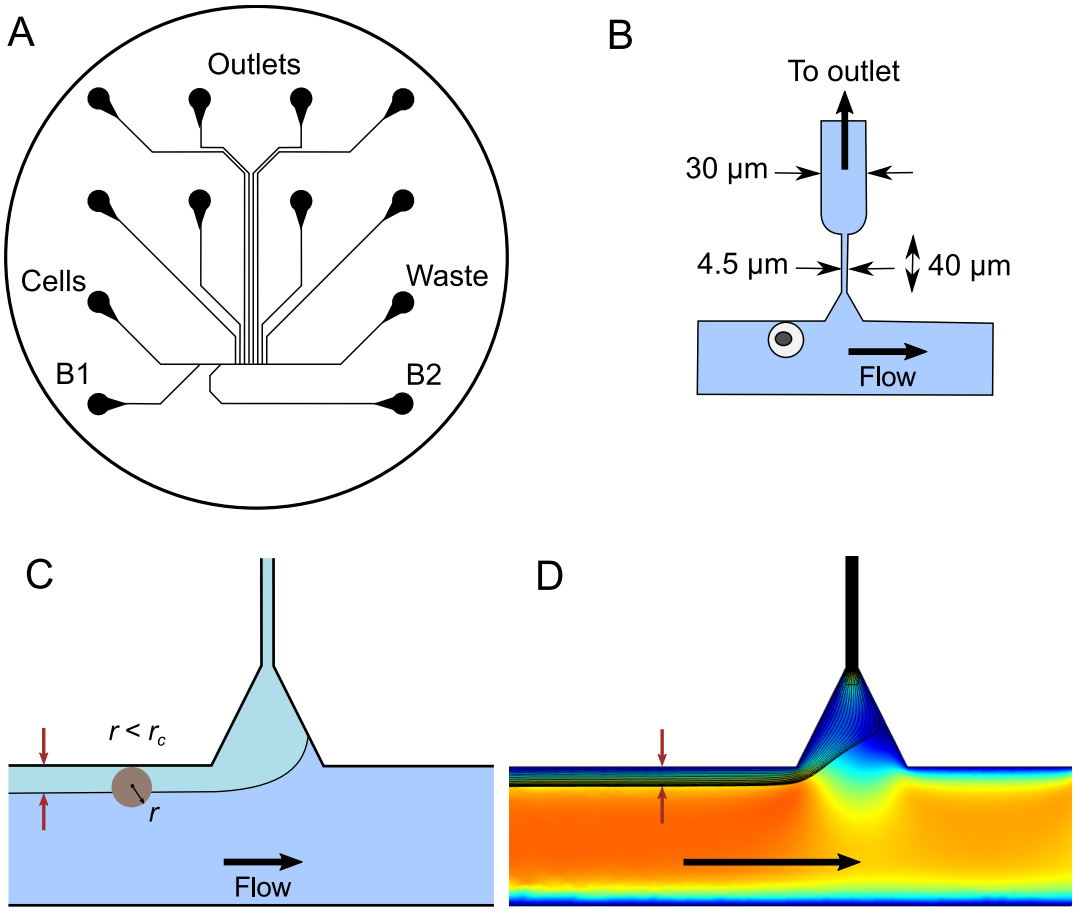

Figure S4. Alternative design of the microfluidic chip for cell trapping with symmetric cell pocket. (A) The overall design with 12 LUER connectors is the same. (B) Cell traps are also 4.5µm-wide but have a symmetric triangular shaped cell pocket. (C) Microfluidic conditions for cell trapping is $r < r_c$. (D) Semi-3D simulation showing the flow velocity as colour code and the streamlines that enter the trap downstream. Same pressure is applied to the cell inlet and the inlet B1 and B2. The lateral position of the outermost streamline entering the trap shows the maximum radius (r_c) a cell can have before it is no longer captured.

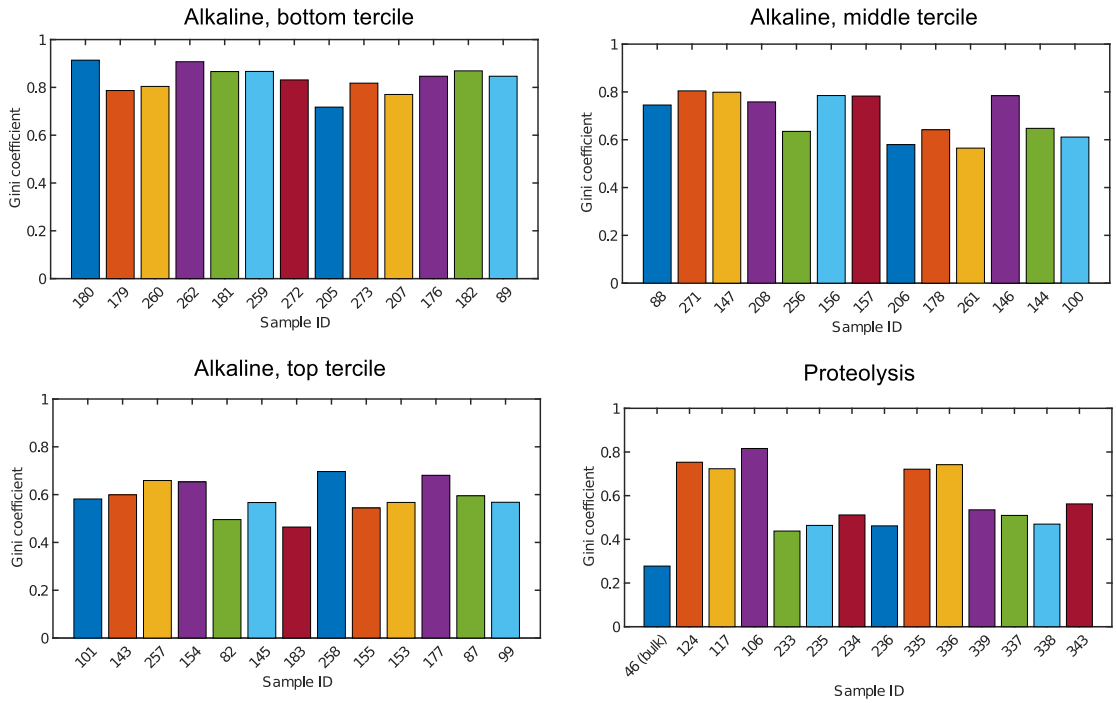

Figure S5. Gini coefficients calculated from the Lorenz plots of cells processed by alkaline lysis belonging to the top, middle and bottom tercile and cells processed by proteolysis.

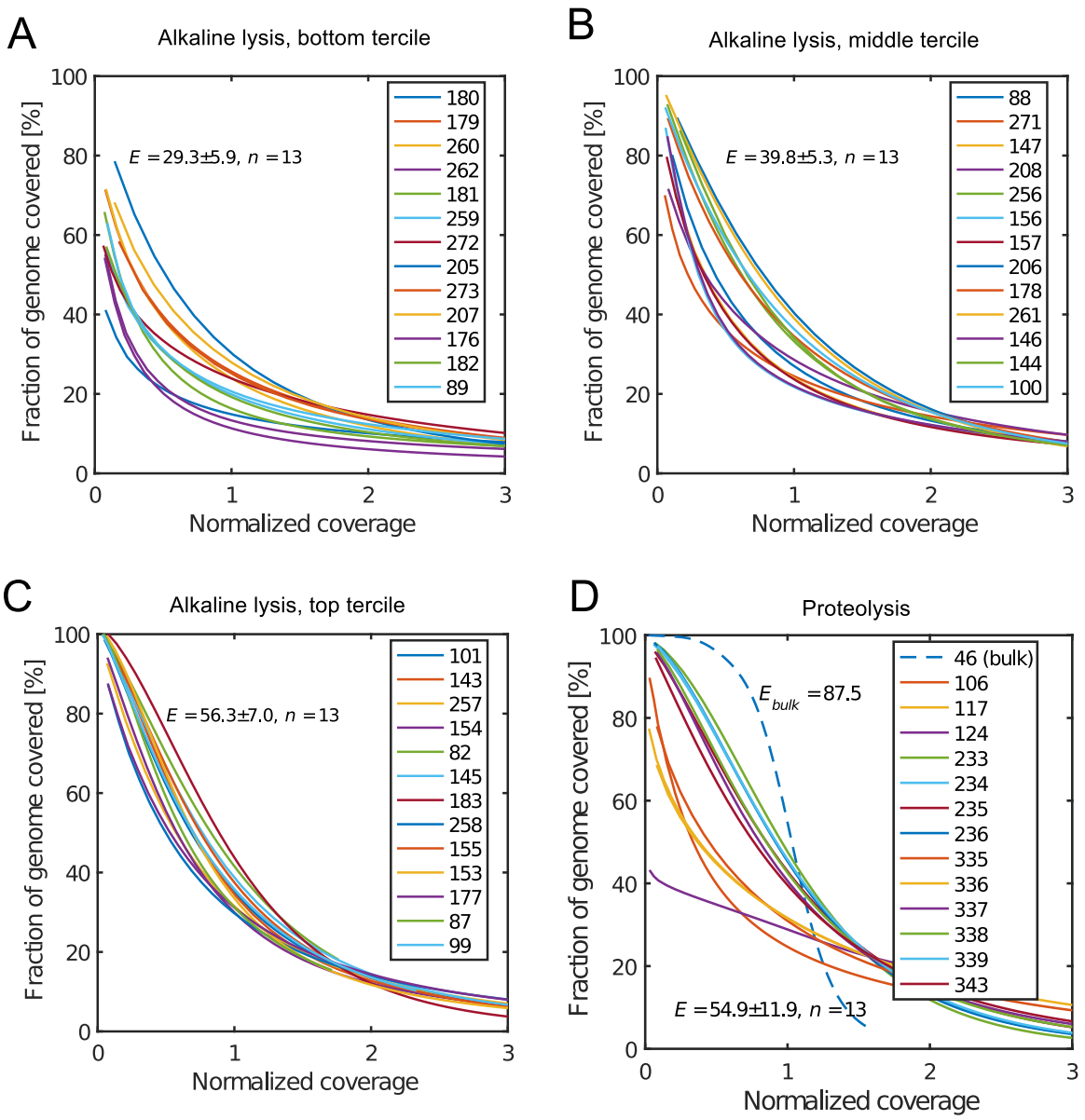

Figure S6. Normalized coverage plots for the LS174T data belonging to (A) the bottom, (B) middle and (C) top terciles of cells processed by alkaline lysis. (D) same for cells processed by proteolysis. Cells 124 to 236 are LS174T cells. Cells 335 to 343 are RKO cells. The mean and SD of the *E*-score is displayed for each group.

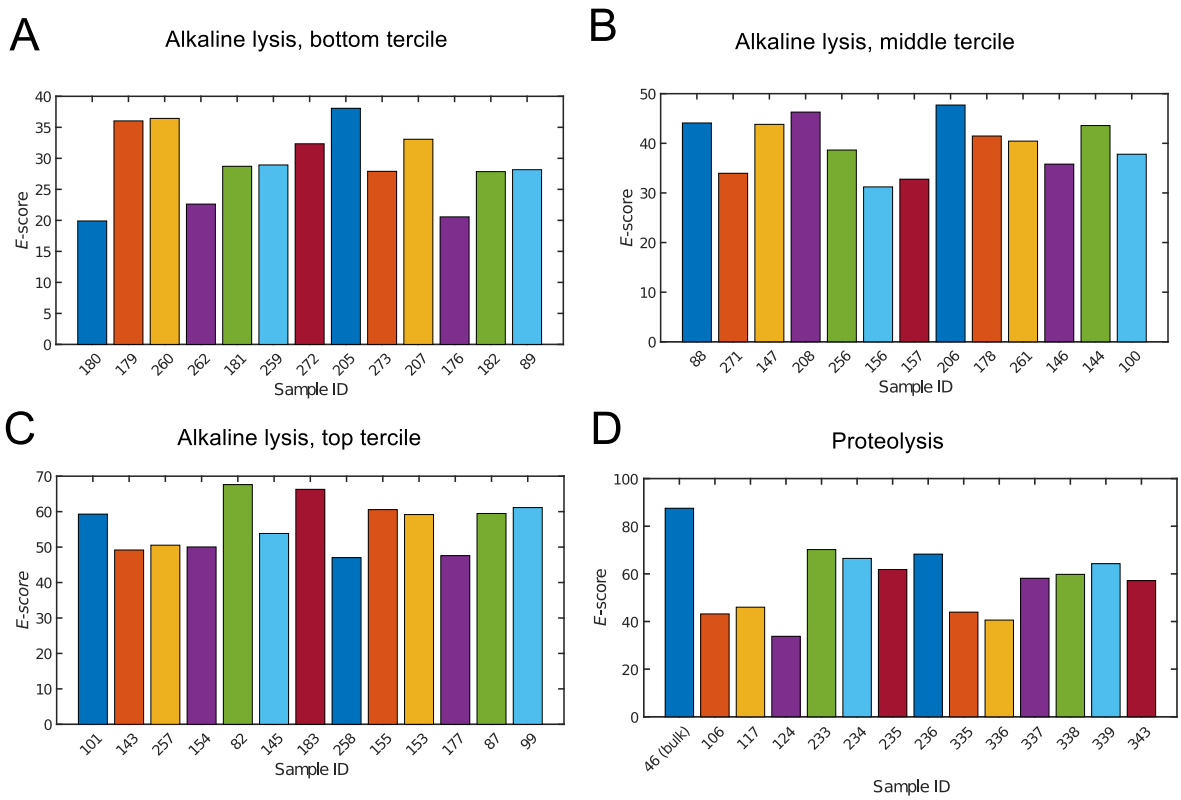

Figure S7. *E*-score of the LS174T data belonging to (A) the bottom, (B) middle and (C) top terciles of cells processed by alkaline lysis. (D) Same for cells processed by proteolysis. Cells 124 to 236 are LS174T cells. Cells 335 to 343 are RKO cells.

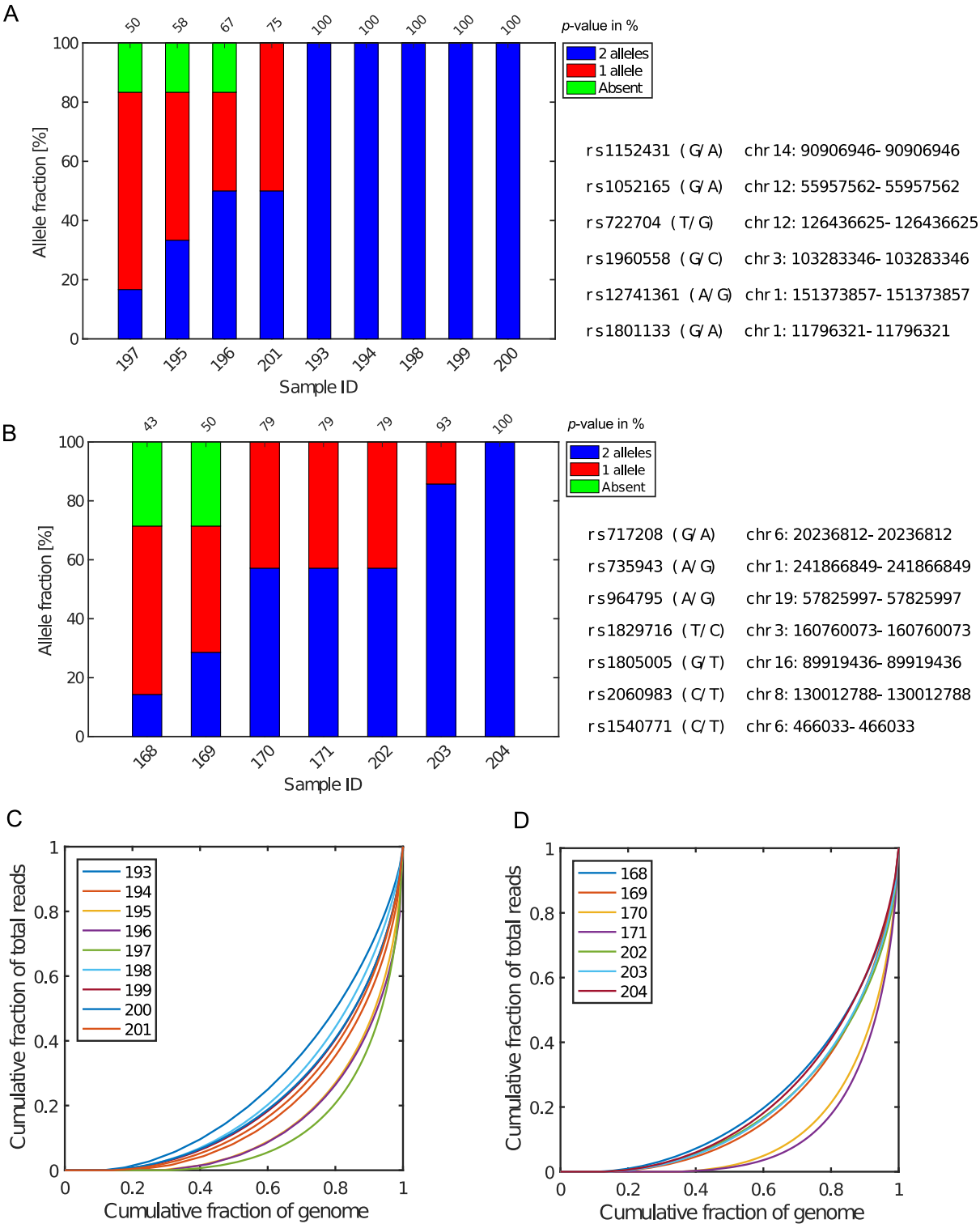

Figure S8. Single cell sequencing of two fresh colorectal cancers. Extent of allelic drop out for single cells analysed directly from two different fresh colorectal cancers (A and B). We display the SNPs used for each sample and the corresponding Lorenz plots (C and D). The fresh tumour samples were obtained with informed consent and the project was approved by the local ethics committee (07/H0606/120).

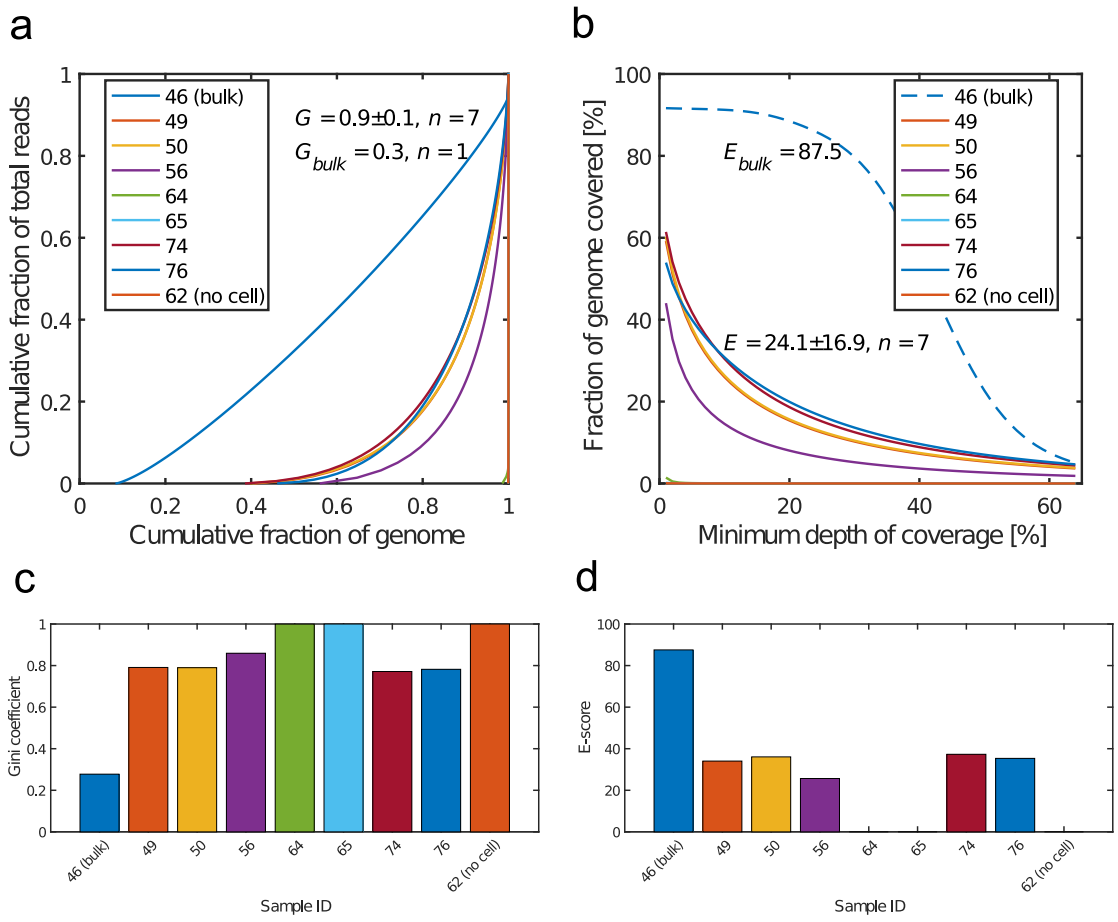

Figure S19. Sequencing of single cells lysed and amplified in PCR tubes. We show the data of seven LS174T single cells sorted by FACS in individual tubes and amplified by MDA following the commercial kit (A). We also display a negative control ('no cell') and the bulk sequencing ('bulk'). (A) Lorenz plots. (B) Coverage plots. (C) *Gini* coefficient. (D) Evenness score.

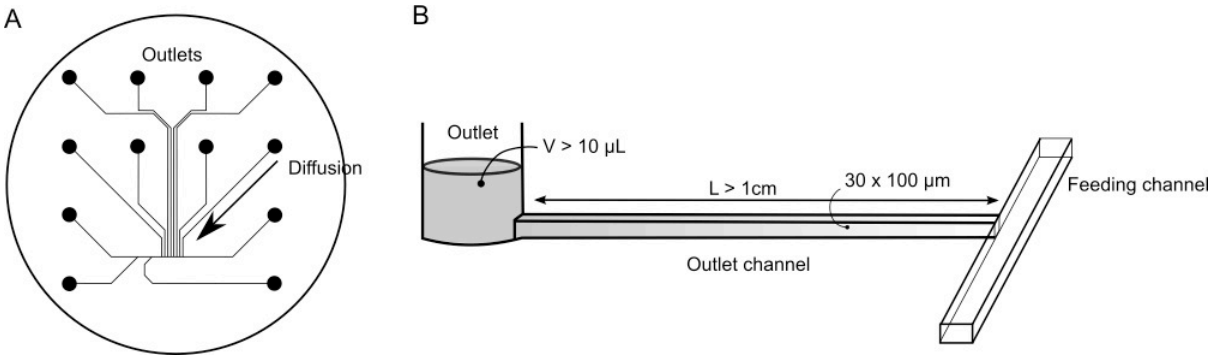

Figure S10. Diffusion from the outlets through the outlet channel during amplification. (A) overall design of the device showing the outlets where amplification is performed. (B) We estimate the loss of material from the outlet well through diffusion into the outlet channel towards the feeding channel.

RKO SNPs	
rs16942 (T/C)	chr17:43091983-43091983
rs234693 (G/C)	chr21:43012770
rs603965 (A/G)	chr11:69648142
rs735943 (A/G)	chr1:241866849-241866849
rs884143 A/C)	chr10:85100317
rs964795 (A/G)	chr19:57825997
rs1424189 (C/T)	chr16:83297005
rs1431759 (A/G)	chr8:87157469-87157469
rs1485682 (A/G)	chrX:66595749
rs2041990 (A/G)	chr19:46147067
rs2059956 (C/T)	chr15:33831331
rs2060983 (A/G)	chr8:130012788
rs12741361 (A/G)	chr1:151373857

LS174T SNPs	
rs735943 (A/G)	chr1:241866849-241866849
rs1431759 (A/G)	chr8:87157469-87157469
rs1801133 (G/A)	chr1:11796321-11796321
rs1473652 (T/C)	chr12:32651038-32651038
rs7853989 (G/C)	chr9:133256205-133256205
rs16942 (T/C)	chr17:43091983-43091983
rs869537 (C/T)	chr4:153034788-153034788
rs36225073 (G/A)	chr11:69639508-69639508
rs717208 (G/A)	chr6:20236812-20236812
rs2059956 (C/T)	chr15:33831331-33831331
rs1667394 (C/T)	chr15:28285036-28285036
rs1583355 (C/T)	chr4:188600283-188600283

Table S2. Selected SNPs for the RKO and LS174T cell line.

Genomic position	Symbol	Chr.	106	117	124	233	234	235	236	335	336	337	338	339	342	Average reads/gene
ENSG00000129864	VCY	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000172288	CDY1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000172468	HSFY1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000169789	PRY	Y	0	1	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000183753	BPY2	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000243643	TSPY20P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000225685	TSPY5P	Y	2	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000236435	TSPY12P	Y	1	0	31	0	0	0	0	0	0	0	0	0	0	
ENSG00000236786	TSPY15P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000188656	TSPY7P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000231874	TSPY18P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000232620	TSPY17P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000234583	TSPY19P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000229940	TSPY22P	Y	0	0	1	0	0	0	0	0	0	0	0	0	0	
ENSG00000168757	TSPY2	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000234110	TSPY25P	Y	0	0	75	0	0	0	0	0	0	0	0	0	0	
ENSG00000228927	TSPY3	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000238074	TSPY6P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000236424	TSPY10	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000215601	TSPY24P	Y	5	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000229159	TSPY23P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000226223	TSPY16P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000238235	TSPY11P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000237902	TSPY21P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000233803	TSPY4	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000187657	TSPY13P	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000229549	TSPY8	Y	1	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000169849	TSPY14P	Y	3	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000258992	TSPY1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000242389	RBMY1E	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000226941	RBMY1J	Y	28	15	9	1	4	5	2	3	5	4	2	1	6	
ENSG00000169800	RBMY1F	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000197038	RBMY1A3P	Y	1	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000229234	RBMY1KP	Y	0	0	4	0	0	0	0	0	0	0	0	0	0	
ENSG00000234414	RBMY1A1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000242875	RBMY1B	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000169811	RBMY1HP	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000244395	RBMY1D	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000205916	DAZ4	Y	0	0	0	0	0	0	0	0	1	0	0	0	0	
ENSG00000188120	DAZ1	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
ENSG00000187191	DAZ3	Y	0	0	0	0	1	0	0	0	0	0	0	0	1	
ENSG00000205944	DAZ2	Y	0	1	1	1	1	1	0	0	0	1	0	1	1	
ENSG00000250868	AC007742.7*	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	
Average number reads/male specfic gene		Y	0,95	0,40	2,81	0,05	0,14	0,14	0,05	0,07	0,14	0,12	0,05	0,05	0,19	**0,40**
Mean nr of reads/gene on X chromosome		X	1192	1933	1262	569	702	691	785	714	581	824	770	718	992	**902**

* AC007742.7 become known as XKRY1 in GRCh38

Table S3. *Data on contamination levels derived by reads on male-specific genes*. The Table lists for the 13 single cell samples studied in Eindhoven (7 from the LS174T cell line and 6 from the RKO cell line [4]) the number of reads found on male-specific genes without a homologue on the Y-chromosome [x}. Mostly zero reads are found to be mapping to these genes, indicating no contamination with exogenous male DNA. For reference the bottom row list the average number of reads/gene found on the X-chromosome in these samples. The average value of this corresponds to 902 reads/gene. This value is to be compare to the average reeds mapping per male-specific gene listed in the before last row. The average value over all samples is 0.4 read.