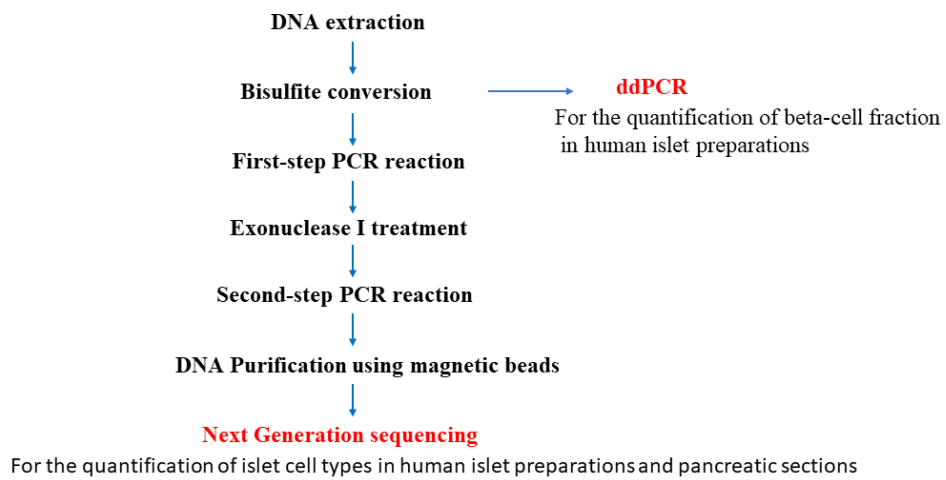


DNA methylation-based assays for the quantification of islet cell-types in human samples

Assay workflow

DNA methylation-based assays for the quantification of islet cell-types: assay workflow



Key Resource Table

Reagent or Resource	Source	Cat. Number (website)
Dneasy® Blood & Tissue Kit	Qiagen	59504 https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit
QIAamp DNA FFPE Tissue Kit	Qiagen	56404 https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-ffpe-tissue-kit
Qubit™ dsDNA Quantification Assay Kit	Invitrogen™	Q32851 https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf
EZ DNA Methylation-Gold	Zymo Research	D5005 https://zymoresearch.eu/collections/ez-dna-methylation-gold-kits/products/ez-dna-methylation-gold-kit
Multiplex PCR Kit	Qiagen	206143 https://www.qiagen.com/us/products/discovery-and-translational-research/pcr-qpcr-dpcr/pcr-enzymes-and-kits/end-point-pcr/qiagen-multiplex-pcr-kit
Exonuclease I	Thermo Scientific™	EN0581 https://www.thermofisher.com/order/catalog/product/EN0581
Kapa pure beads		07983271001 https://elabdoc-prod.roche.com/eLD/web/pi/en/products/SEQ-KAPA-0161

Targeted Bisulfite Sequencing protocol

DNA extraction

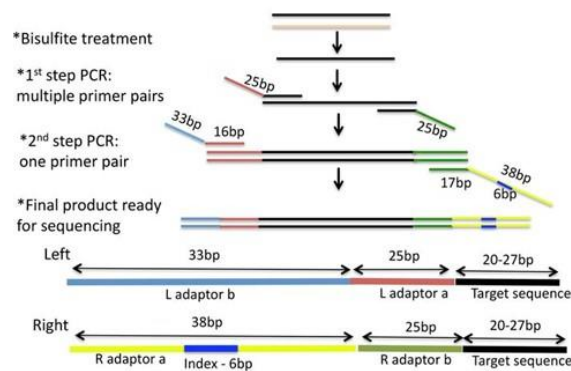
DNA from sorted cells or islets (up to 5×10^6 cells) is extracted using the Qiagen Dneasy® Blood & Tissue Kit (Cat.: 59504). DNA extraction from Formalin-Fixed Paraffin-Embedded (FFPE) pancreatic tissue sections is carried out using the Qiagen QIAamp® DNA FFPE Tissue Kit (Cat.: 56404). DNA extraction is performed according to manufacturer's instructions, with slight modifications: cell lysis is performed at 56°C for 1h and extracted DNA is eluted with two consecutive volumes of 20 µl water (total elution volume: 40 µl) and stored at -20°C. DNA concentration is measured using a Qubit fluorometer (Qubit™ dsDNA Quantification Assay Kit, Cat.: Q32851).

Bisulfite conversion reaction

Bisulfite treatment is performed on DNA from pancreatic islets (50ng DNA per reaction) or from pancreatic sections (200ng per reaction) using the Zymo EZ DNA methylation-Gold™ Kit (Cat.: D5005) and the recommended protocol. The preparation of CT conversion reagent includes the addition of 600µl water, 300µl M-Dilution buffer and 50 microliter M-Dissolving Buffer. We add 100µl of the CT Conversion Reagent to 50-55µl of DNA. Converted DNA is eluted with two consecutive volumes of 12µl water and stored at -20°C.

First-step PCR

To amplify and sequence multiple methylation markers from bisulfite-treated genomic DNA, we use a two-step multiplexed PCR protocol (**Figure 1**; JCI Insight. 2020;5(14):e136579. <https://doi.org/10.1172/jci.insight.136579>). Genome coordinates for each cell-type specific methylation marker and primer sequences (including a 5'-end 25-mer Illumina TruSeq universal adapter without index tags) are listed in **Supplementary Table 4**.



From Neiman D. et al. JCI Insight. 2020;5(14):e136579. <https://doi.org/10.1172/jci.insight.136579>.

Figure 1: Scheme describing the procedure for multiplex amplification of methylation markers.

A primer mix is prepared as outlined in **Table 1** achieving a final volume of **250µl** with TE (TRIS-EDTA).

Table 1: Primer mix preparation

Cell type	Marker	Forward primer (100µM)	Reverse primer (100µM)	Primers final concentration in 250 µl mix
Alpha-cells	PDX1	10µl	10µl	4 µM
Alpha-cells	CCDC73	10µl	10µl	4 µM
Alpha-cells	PELI2	5µl	5µl	2 µM
Beta-cells	Insulin	5µl	5µl	2 µM
Beta-cells	FBXL19	5µl	5µl	2 µM
Beta-cells	DLG5	5µl	5µl	2 µM
Delta-cells	ZBTB7C	5µl	5µl	2 µM
Delta-cells	RPGRIP1L	5µl	5µl	2 µM
Delta-cells	UBLCP1	10µl	10µl	4 µM
Acinar cells	PTPRE	5µl	5µl	2 µM
Acinar cells	PRDM2	5µl	5µl	2 µM
Acinar cells	GRB10	5µl	5µl	2 µM
Acinar cells	FBXW12	5µl	5µl	2 µM
Acinar cells	SPATA21	5µl	5µl	2 µM
Ductal cells	BZW2	5µl	5µl	2 µM
Ductal cells	COL27A1	5µl	5µl	2 µM

Ductal cells	HUNK	5µl	5µl	2 µM
Endothelial cells	RB1	5µl	5µl	2 µM
Endothelial cells	CMIP	5µl	5µl	2 µM
Endothelial cells	ST5	5µl	5µl	2 µM

For each sample, the PCR reaction (total volume: 25µl) is prepared in TempAssure 0.2mL PCR 8-Tube Strips (Att. Optical caps, USA scientific, Cat.: 1402-4700) using the QIAGEN Multiplex PCR Kit (Cat.: 206143) according to the manufacturer's instructions with 5µL of bisulfite-treated DNA, as detailed in **Table 2**.

Table 2: Reagents for first-step PCR reaction

Reagent	Final concentration	Amount
Primer mix	0.4 or 0.2 µM	2.5 µl
2X Multiplex PCR Master Mix	1X	12.5µl
Molecular-grade water	N/A	5 µl
DNA	N/A	5 µl
Total		25 µl

The first-step PCR cycling protocol is detailed below (**Table 3**).

Table 3: First-step PCR cycling conditions

Step	Temperature	Time	Cycles
Denaturation	95°C	15 min	1
Denaturation	95°C	30 s	30
Annealing	57°C	3 min	
Extension	72°C	1.5 min	
Final extension	68°C	10 min	1
Hold	4°C	Indefinitely	

Products from first-step PCR reaction can be used immediately for Exonuclease I treatment and second-step PCR reaction or stored at 4°C for later use.

Exonuclease I treatment

Products from first-step PCR reaction are first treated with Exonuclease I to remove excess primers. 4.5 µl of Exonuclease I reaction mix and buffer (Thermo Fisher Scientific, Cat.: EN0581) is prepared and added to each first-step PCR reaction tube (**Table 4**).

Table 4: Exonuclease I reaction

Reagent	Amount
First-step PCR product	25 µl
Exonuclease I reaction mix	2 µl
Buffer X10	2.5 µl
Total	29.5 µl

Incubation is at 37°C for 45 minutes, followed by a 15-minute incubation at 85°C for enzyme inactivation.

Second-step PCR

The second PCR step is prepared using primers that elongate the full TruSeq universal adaptor sequences. The forward primer is uniform across all reactions, while the reverse primer incorporates a distinctive 6-base pair index barcode for each sample, facilitating sample pooling for multiplex Illumina sequencing (see Supplementary Table 4 and Scheme).

For each sample, the second-step PCR reaction is prepared in 0.2mL PCR 8-Tube Strips, using 2x PCRBIO HS Taq Mix Red (PCR Biosystems, Cat.: PB10.23-10) as detailed below (**Table 5**).

Table 5: Reagents for second-step PCR reaction

Reagent	Final concentration	Amount
2x PCRBIO HS Taq Mix Red	1X	12.5 µl
Primers (10 µM)	0.4 µM	1 µl
DNA (from 1st-step PCR after Exonuclease I reaction)	N/A	11.5 µl

Use second-step PCR cycling protocol (**Table 6**)

Table 6: Second-step PCR cycling conditions

Step	Temperature	Time	Cycles
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Denaturation	95°C	2 min	1
Denaturation	95°C	30 s	15
Annealing	59°C	1.5 min	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	Indefinitely	

DNA cleanup

The PCR samples are then pooled (pool up to 40 samples) and cleaned using Kapa pure beads (Roche, Cat.: 07983271001), according to the manufacturer's instructions for 0.8X with the following modifications:

- DNA from second-step PCR reaction is cleaned up in a minimal volume of 50 µl: if cleanup is performed on only one PCR reaction (25 µl) add 25 µl of 10 mM Tris-HCl (pH 8.0 – 8.5) to reach a 50 µl volume. If 2 samples or more are pooled (volume ≥ 50 µl), use 50 µl from pooled samples for cleanup and keep leftover at -20°C.
- 40µl of Kapa Pure Beads is added per 50µl DNA.
- Beads are incubated for 30 min at room temperature before use.
- Elution is in 50µl elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5), elution time: 10 min.
- The whole cleaning procedure is repeated once more with 40µl of Kapa Pure Beads added to the 50µl cleaned supernatant. The final elution is performed in 30µl elution buffer.

Next-Generation sequencing and analysis

Library concentration is measured using Qubit® Fluorometer (Invitrogen, Thermo Fisher Scientific) and quality control assessed using TapeStation capillary electrophoresis (Agilent, Santa Clara, CA, USA). The library is sequenced on NextSeq 500/550 (Mid Output Kit, Illumina, Inc., 150bp single reads).

Sequenced reads were separated by barcode, and aligned to the target sequence with Bismark, using a computational pipeline available (<https://github.com/Joshmoss11/btseq>). Reads are quality filtered based on Illumina quality scores and identified as having at least 80% similarity to the target sequences and containing all the expected CpGs. Samples with less than 1000 total reads are filtered-out.

CpGs are considered methylated if “CG” is read, and unmethylated if “TG” is read. Proper bisulfite conversion is assessed by analyzing methylation of non-CpG cytosines.

For each marker (except for PDX1 and ST5) the fraction of molecules wherein all CpG sites are unmethylated is calculated.

Conversely for PDX1 and ST5 markers, the fraction in which all CpG sites are methylated is determined.

Final quantification includes correction for assay efficiency based on analysis of spike-in experiments.

Table 7 provides the linear regression equation for each marker and cell type to quantify cell fractions within the range of 10% to 100% (e.g., in islet preparation):

Table 7: Linear regression equations for the estimation of cell fractions in islet preparations

Marker	cell type	Equation
PDX1	Alpha-cells	$y = 1.5x - 3$
CCDC73	Alpha-cells	$y = 1.2x - 2.3$
PELI2	Alpha-cells	$y = 1.2x - 1.5$
Insulin	Beta-cells	$y = 1.3x - 0.6$
FBXL19	Beta-cells	$y = 1.2x + 0.1$
DLG5	Beta-cells	$y = 1.3x + 3.1$
ZBTB7C	Delta-cells	$y = 1.4x + 0.3$
RPGRIP1L	Delta-cells	$y = 1.3x - 0.1$
UBLCP1	Delta-cells	$y = 6.7x + 0.1$
PTPRE	Acinar cells	$y = 1.2x + 0.04$
PRDM2	Acinar cells	$y = 1.1x + 1.7$
GRB10	Acinar cells	$y = 1.3x + 0.3$
FBXW12	Acinar cells	$y = 1.1x + 1.7$
SPATA21	Acinar cells	$y = 1.4x + 0.9$
BZW2	Ductal cells	$y = 2x - 0.2$
COL27A1	Ductal cells	$y = 1.4x$
HUNK	Ductal cells	$y = 1.6x - 0.1$
RB1	Endothelial cells	$y = 1.2x - 0.5267$
CMIP	Endothelial cells	$y = 4.4x - 0.1$
ST5	Endothelial cells	$y = 2.3x - 0.02$

(x: fraction as determined from sequencing analysis; y: corrected fraction)

Table 8 provides the linear regression equations to quantify alpha and beta cell fractions within the range of 0% to 3% (e.g., in pancreatic sections):

Table 8: Linear regression equations for the estimation of alpha and beta cell fractions in pancreatic sections

Marker	cell type	Equation
PDX1	Alpha-cells	$y = 0.4x + 0.04$
CCDC73	Alpha-cells	$y = 0.97x - 0.03$
PELI2	Alpha-cells	$y = x - 0.1$
Insulin	Beta-cells	$y = 1.1x - 0.1$
FBXL19	Beta-cells	$y = 1.7x + 0.01$
DLG5	Beta-cells	$y = 1.1x + 0.1$

(x: fraction as determined from sequencing analysis; y: corrected fraction)

For each sample, the average cell fraction is determined by calculating the mean of the results obtained from individual markers corresponding to each cell type.

Bisulfite ddPCR (Droplet Digital PCR)-based quantification of beta-cell fraction in human islets

DNA is extracted from islet preparations, bisulfite treatment performed on 20ng islet DNA and converted DNA is eluted with two consecutive volumes of 12µl water (final volume: 24µl), as described above in the Targeted Bisulfite Sequencing Protocol.

ddPCR reactions are performed using the QX200 Droplet Digital PCR System from Bio-Rad: Reference manuals for the QX200 Droplet generators, PX1 PCR plate sealer, QX200 Droplet reader, and accompanying software are available from the Bio-Rad website (<https://www.bio-rad.com/en-il/life-science/digital-pcr/qx200-droplet-digital-pcr-system>)

The insulin gene amplicon, primers and probes used for beta-cell fraction quantification in human islet preparations are detailed in Supplementary Table 4.

The ddPCR reaction mix is prepared as detailed in **Table 9** and distributed in PCR 8-well strip tubes.

Table 9: Reagents of ddPCR reaction

Probe (Dye)/Reagent (stock concentration)	Final concentration	Amount for single reaction
all DNA sense probe (HEX) (250nM)	25nM	2.2µl
Unmethylated Antisense Probe (FAM) (250nM)	25nM	2.2µl
Primers (Reverse and Forward) (10µM, each)	1µM, each	2.2µl
Molecular-grade water	N/A	1.4µl
Supermix for probes (no dUTP) (2×)	1X	11µl
DNA template	N/A	3µl
Total		22µl

Each DNA sample is assayed in duplicates and a no DNA template control is included.

Droplet generation and thermal-cycling incubation

For each 8-sample PCR strip:

1. A DG8 Droplet Generator Cartridge for droplet generation is inserted into plastic cartridge holders.
2. Using a multi-channel pipette, 20 µL of reaction mix from a PCR strip are slowly dispensed to the middle row of wells on the cartridge.
3. 70 µL of Droplet generation oil for probes are transferred to the bottom row of wells on the cartridge.

4. A DG8 gasket is applied to the cartridge. The cartridge is then inserted into QX200 Droplet Generator.
5. Following droplet generation, 40 μ L of droplets are transferred using a multi-channel pipette, to the first column of wells on a ddPCR 96-well plate.
6. Steps 1-5 are repeated for additional samples
7. The droplet-containing plate is heat-sealed with PCR Plate Heat Seal pierceable foil.

Droplets are incubated on a thermal cycler using the following PCR cycling protocol (**Table 10**)

Step	Temperature	Time	Cycles
Denaturation	95°C	10 min	1
Denaturation	95°C	30 s	35
Annealing and extension	53.8°C	1 min	
Enzyme inactivation	98°C	10 min	

The droplet-containing plate is loaded into a QX200 Droplet reader.

Analysis

QuantaSoftTM software (version 1.7.4) is used to assign positive/negative droplets.

Thresholds are manually set for each sample using acceptance criteria defined during the optimization of each experiment.

Samples with less than 10,000 accepted droplets are excluded.

Fraction of beta-cells= copies amplified by unmethylated probes/ copies amplified by All DNA probe.