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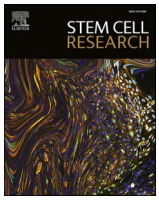

Lab Resource: Multiple Cell Lines

Generation of two human induced pluripotent stem cell lines from a patient with Neurofibromatosis type 1 (NF1) and pathogenic *NF1* gene variant c.1466 A>G BCRTi011-A as well as a first-degree healthy relative (BCRTi010-A)

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A B S T R A C T

We describe the generation of two human induced pluripotent stem cell (iPSC) lines derived from peripheral blood mononuclear cells (PBMCs) using a non-integrative episomal reprogramming strategy. The first cell line was derived from a NF1 patient with the genetic variant c.1466A>G (BCRTi011-A) which leads to a cryptic splice site and aberrant splicing. The second one was created from a healthy relative of first-degree (BCRTi010-A). The generated iPSC lines were shown to have tri-lineage differentiation potential, a normal karyotype, and expression of pluripotent markers. Both iPSC lines provide a powerful tool for *in vitro* disease modeling and therapy development.

1. Resource Table

Unique stem cell lines identifier	BCRTi010-A https://hpscreg.eu/cell-line/BCRTi010-ABCRTi011-Ahttps://hpscreg.eu/cell-line/BCRTi011-A
Alternative name(s) of stem cell lines	N/A
Institution	Charité - Universitätsmedizin Berlin, Berlin BIH-Center for Regenerative Therapies (BCRT)
Contact information of distributor	BIH Center for Regenerative Therapies (BCRT) Andreas Kurtz, Andreas.kurtz@bih-charite.de
Type of cell lines	hiPSC
Origin	Human
Additional origin info required *for human ESC or iPSC*	Age: 42 (BCRTi010-A); Age: 12 (BCRTi011-A) Sex: male (BCRTi010-A), female (BCRti011-A), Ethnicity: European
Cell Source	PBMCs
Clonality	Clonal
Method of reprogramming	Episomal (OCT3/4, SOX2, L-MYC, KLF4)
Genetic Modification	N/A
Type of Genetic Modification	N/A
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Associated disease	Neurofibromatosis Type 1 (NF1)
Gene/locus	Chr 17: 26.45 – 26.73 Mb, NM_000267, NP_000258 ENSG00000196712. BCRTi010-A: no *NF1* sequence alteration.

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[2] Both authors share senior authorship.

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(*continued*)

	BCRTi011-A: heterozygous *NF1* sequence alteration (mutation) c.1466A>G; p. Tyr489Cys (Y489C), g. chr17:29541542A>G (ClinVar: VCV000000354.73); additionally *NF1*-VUS c.1954C>T, p.Arg652Cys, g. chr17:29552221C>T (ClinVar: VCV000185762.11).No other sequence alterations in a tumor disease panel of 403 genes and of 32 rasopathy gene panels (at least type C) in both cell lines.
Date archived/stock date	2022
Cell line repository/bank	Human Pluripotent Stem Cell Registry ((hPSCreg®) https://hpscreg.eu/cell-line/BCRTi010-A https://hpscreg.eu/cell-line/BCRTi011-A
Ethical approval	This work was approved by the Ethics Commission of the Charité – Universitätsmedizin Berlin (EA4/110/10) and the Ethics Commission of Martin Luther University (2021–177, 10.1.2022).

2. Resource utility

NF1 is a common autosomal dominant genetic disorder (OMIM 162200) caused by pathogenic germline variants of the *NF1* gene (OMIM 613113) on chromosome 17q11.2. The hiPSC lines (BCRTi010-A, BCRTi011-A) from a healthy relative and a NF1 patient of the same family offer a useful resource for disease modeling and therapy development.

3. Resource details

NF1 is an autosomal dominant tumor predisposition syndrome with an approximate incidence of 1:3500 (Rasmussen and Friedman, 2000). About 50% of affected individuals show pathogenic germline variants of the highly conserved *NF1* gene. NF1 patients are at risk to develop brain tumors, malignant peripheral nerve sheath tumors, breast cancer, and other malignancies (Uusitalo et al., 2016). One main function of the protein neurofibromin is to act as a GTPase-activating protein and negatively regulate Ras. It is composed of several domains and acts in an auto-inhibited or functional state (Chaker-Margot et al., 2022). Until now there is no curative therapy for this genetic disease.

PBMCs were reprogrammed with commercially available Epi5™ Episomal iPSC Reprogramming Kit (Thermo Fisher Scientific). BCRTi010-A and BCRTi011-A lines were derived from a single colony that was isolated after reprogramming and subsequently characterized (Table 1). Both generated cell lines showed the typical morphology of undifferentiated hiPSC when maintained in Essential 8 (E8, STEMCELL Technologies) medium (Fig. 1A). A master cell bank was generated and subsequently characterized. Pluripotency of derived hiPSc lines was confirmed through the detection of octamer-binding transcription factor 3/4 (OCT3/4), NANOG and Sex determining region Y-box 2 (SOX2), tumor rejection antigen (TRA-1-60), and stage-specific embryonic antigen 4 (SSEA4) by fluorescence microscopy (Fig. 1B). Flow cytometric analysis showed expression of pluripotency markers at passage 13 (Fig. 1C and D). The absence of episomal vectors was confirmed by RT-PCR of BCRTi010-A in passage 10 and of BCRTi011-A in passage 12 (Fig. 1E). To proof the pluripotency differentiation potential, embryoid bodies were formed from both hiPSC lines and the differentiation into endodermal (Goosecoid, Sox17), mesodermal (Brachyury and MIXL1), and ectodermal cells (NESTIN and NEUROD) were confirmed by RT-PCR (Fig. 1F). The karyotypes of the cell lines were tested by SNP analysis and showed normal karyotypes for both cell lines (Supplementary Fig. 1). The identity of both lines was confirmed by short tandem repeat (STR) analysis of 16 genomic loci including AMEL, CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, Penta_D, Penta_E, TH01, TPOX, and vWA. Identical DNA profiles between the patient's PBMCs and generated hiPSC lines were observed (Supplementary Table S1 and S2). Moreover, tests were negative for mycoplasma contamination as well as for HIV 1 + 2, Hepatitis B, and Hepatitis C.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase contrast microscopy	*Normal PSC morphology*	Fig. 1*A*
Phenotype	*Immunocytochemistry*	*Positive staining for OCT3/4, NANOG, SOX2, TRA 1–60, SSEA-4*	Fig. 1*B*
	Quantitative analysis *(i.e. Immunocytochemistry counting, Flow cytometry, RT-qPCR)*	*BCRTi010-A* *OCT3/4: (80.7%), TRA 1–60: (93.1%), SSEA-4: (96.3%),SOX2: (99%)* *BCRTi011-A* *OCT3/4: (89.2%), TRA 1–60: (97.7%), SSEA-4: (99.9%),SOX2: (99.1%)*	Fig. 1 *C and D*
Genotype	Karyotype (SNP array)	Normal Karyotype Resolution 700,000 markers	*Supplementary Fig. S1 and S2*
Identity	Microsatellite PCR (mPCR) OR	*DNA Profiling Not performed*	
	STR analysis	16 STR sites were analyzed, all matching between donor urinary cells and reprogrammed hiPSC lines	*Supplementary Table. S1 and S2*
Mutation analysis (IF APPLICABLE)	Sequencing	*NGS*	
	Southern Blot OR WGS	Illumina NovaSeq6000	
Microbiology and virology	Mycoplasma HIV 1 + 2 Hepatitis B, Hepatitis C	*Negative*	Not shown but available with the author
Differentiation potential	Spontaneous *in vitro* differentiation and RT PCR	Germ layer-specific marker expression:ectoderm (Nestin and NeuroD), mesoderm (Brachyury and MIXL1) , and endoderm (Gooscoid and SOX17	Fig. 1*F*
List of recommended germ layer markers	RT PCR	Germ layer-specific marker expression by RT PCR:ectoderm (Nestin and NeuroD), mesoderm (Brachyury and MIXL1), and endoderm (Gooscoid and SOX17)	Fig. 1*F*
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	*Negative*	*Not shown but available with the author*
Genotype additional info (OPTIONAL)	Blood group genotyping	*N/A*	
	HLA tissue typing	*N/A*	

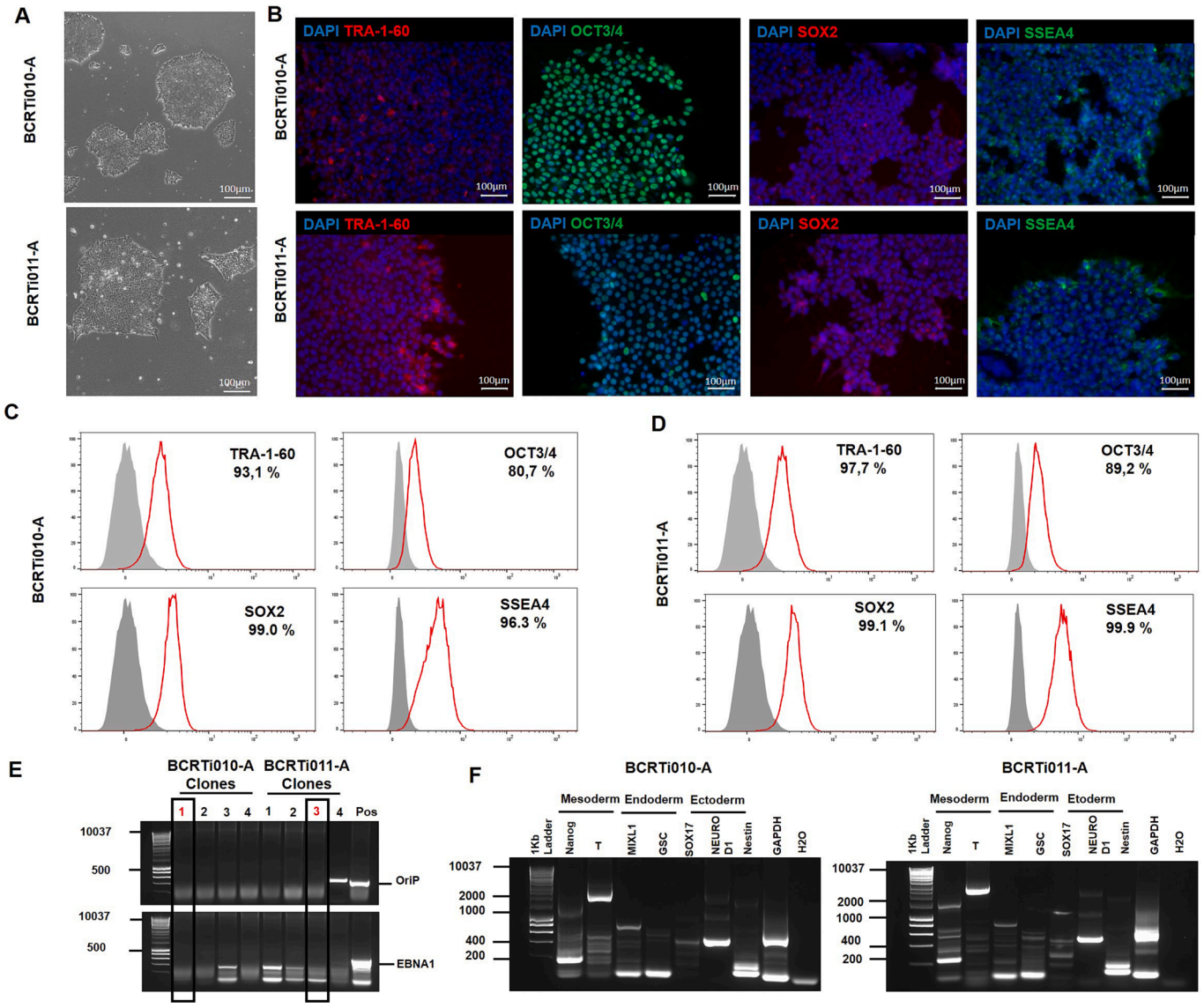

Fig. 1.

4. Materials and methods

4.1. Reprogramming

Collected blood samples from donors were used to isolate PBMCs by density gradient centrifugation with Biocoll® Trennlösung (Bio&SELL). PBMCs were maintained for five days in StemPro®-34 SFM medium (Gibco) supplemented with 100 U/mL Pen-Strep (P/S, Biochrom), 1% GlutaMAX™, 100 ng/mL SCF and Flt-3 L, 20 ng/mL IL-3 and 10 IL-6 ng/mL (all Gibco). Reprogramming was performed with Epi5™ Episomal iPSC Reprogramming Kit (Invitrogen) according to the manufacturer's recommendation. In brief, reprogramming factors (OCT4, SOX2, LIN28, KLF4, and L-MYC) were delivered in episomal vectors oriP/EBNA-1 (Epstein–Barr nuclear antigen-1). Electroporation was performed at 1650 V in three pulses for 10 ms with $5x10^5$ cells in the neon electroporator (Thermo Fischer Scientific). Then, cells were seeded under feeder-free conditions on Geltrex (Life Technologies) matrix-coated 6 well plates (Corning) containing 2 mL of StemPro®-34 medium. Daily medium changes were performed with N2B27 medium, consisting of DMEM/F12 w/ HEPES supplemented with N2 supplement (1×), B27 supplement (1×), MEM-non-essential amino acids (1×), GlutaMAX-1 (1/2×), β-mercaptoethanol (55 µM; all Gibco) and 100 ng/mL bFGF (Peprotech). On day 9 medium was switched to E8. Between days 20 and 25, colonies were picked and transferred to Geltrex coated 24 well plates (Corning) in E8 medium supplemented with 1% P/S and 10 µM Rock Inhibitor Y-27632 (Wako). Cells were EDTA/Clump passaged every 4 to 5 days at a ratio of 1:6. Picked colonies were cryopreserved. MycoAlert™ Mycoplasma detection kit (Lonza) was used to test derived hiPSCs for mycoplasma contamination, while PBMCs were tested for viruses HIV 1 + 2, Hepatitis B and C by external diagnostic laboratories (IDEXX BioAnalytics, Kornwestheim, Germany).

4.2. RT-PCR for the absence of reprogramming vector

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen). TaqMan Reverse Transcription Reagents (Applied Biosystem) were used to generate cDNA. RT-PCR was performed with primers listed in Table 2 and following program: 94 °C for 2 min (1x cycle), 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min (30x cycle), and 72 °C for 7 min (1x cycle). RNA from passage 2 of hiPSC was used as a positive control.

4.3. Detection of pluripotency markers

Both hiPSC lines were fixed, permeabilized, blocked, and stained for

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Oct3/4 A488 SSEA4-FITC TRA-1-60-DyLight Nanog *(purified)*	1:200 1:200 1:100 1:150	BD Biosciences, Cat# 560217 R&D SystemCat# FAB1435P Novous Biologicals Cat# NB100-730R Cell Signalling Technology Cat# 4903S	AB_1645305 AB_357038 AB_11006064 AB_10559205
Secondary antibodies	Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 546	1:1000	Thermo Fisher Scientific Cat# A 10036	AB_2534012

		Primers	
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Episomal Plasmids (RT-PCR)	Orip EBNA-1	*544bp* *666bp*	TTCCACGAGGGTAGTGAACC/ TCG GGGGTGTTAGAGACAAC ATCGTCAAAGCTGCACACAG/ CCCAGGAGTCCCAGTAGTCA
Trilineage markers	*NANOG,* Brachury (T) MIXL1 GSC SOX17 NEUROD1 Nestin	*237bp* *2360bp* *67bp* *89bp* *94bp* *2081bp* *1807bp*	AAGGTCCCGGTCAAGAAACAG/ CTTCTGCGTCACACCATTGC AATTGGTCCAGCCTTGGA/ CGTTGCTCACAGACCACA CTGTTCCCCTCTCTCTGAAGA/ GGCAGAAAAGATGTGTTCCTCC AACGCGGAGAAGTGGAACAAG/ CTGTCCGAGTCCAAATCGC GTGGACCGCACGGAATTTG/ GGAGATTCACACCGGAGTCA GCCCCAGGGTTATGAGACTATCACT/ CCGACAGAGCCCAGATGTAGTTCTT
House-Keeping Genes (qPCR)	GAPDH	393bp	TTGCCATCAATGACCCCTTCA/ CGCCCCACTTGATTTTGGA

pluripotency markers with primary and secondary antibodies stain with donkey mouse-specific A546 (Invitrogen) with Cytofix and Perm/Wash reagents (both BD Bioscience). Cell nuclei staining was done with 4′,6-Diamidin-2-phenylindo (DAPI, Invitrogen), and analysis was performed by microscopy (Axio Observer, Zeiss).

4.4. Flow cytometry

Both HiPSC lines were harvested, washed with PBS, permeabilized with BD Cytofix/Cytoperm™, washed, and kept in BD Perm/Wash Buffer (both BD Bioscience). Cells were incubated with primary conjugated antibodies at 4 °C for 30 min, washed, and measured at MACSQuant (Miltenyi Biotec). Analysis was done with FlowJo v10.2 software (FlowJo™, LLC).

4.5. In vitro trilineage differentiation

Both hiPSC lines were harvested using accutase (Gibco) and seeded into AggreWell plates 800 (Stemcell Technologies) in E8 medium supplemented with 10 μm Rock inhibitor. After 24 h, EBs were transferred to ultra-low attachment plates (Corning) in KO-DMEM medium (Invitrogen) supplemented with 20% Knockout SerumReplacer (Invitrogen), 1% (v/v) NEAA, 0.2% (v/v) beta-Mercaptoethanol, 1.25 mM GlutaMax™, and 1% P/S. Medium change was performed every 48 h. Total RNA was isolated from harvested EBs after 10 days using RNeasy Plus Mini Kit (Qiagen) for RT-PCR. TaqMan Reverse Transcription Reagents (Applied Biosystem) were used to generate cDNA. RT-PCR was performed with primers listed in Table 2 with the program: 96 °C for 5 min, 35 cycles of 96 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, 72 °C for 7 min.

4.6. Karyotype and short tandem repeat (STR) analysis

Karyotyping analysis was performed by LIFE & BRAIN Biomedical & Scientific Technology Platform (genomics@lifeandbrain.com). Karyotyping based on genotyping of 700,000 markers was performed using Illumina microarray. Genomestudio 2.0 software was used for the analysis.

STR analysis was conducted by external diagnostic laboratories IDEXX BioAnalytics, Kornwestheim, Germany). The analysis was performed for hiPSCs and their parental PBMCs with detection of 16 loci.

4.7. Whole genome sequencing and analysis

We undertook targeted next-generation sequencing (NGS) and searched for variants in a set of genes known to be causative for neurofibromatosis and RASopathy-encoded genes. Target enrichment was done by High-throughput NGS - Illumina NovaSeq6000. The in-house pipeline and the human reference genome (Genome Reference Consortium GCA_000001405.1 GCF_000001405.13; HG19) were used to evaluate the data. For sequence analysis we checked 403 genes associated with tumor disease and 32 genes involved in Rasopathies: *ABCA3, ABCB7, ABCG5, ABCG8, ACD, ACTB, ACTN1, ADAMTS13, AIP, AK1, AK2, AKT1, ALAS2, ALK, AMN, ANK1, ANKRD26, AP3B1, AP3D1, APC, ARPC1B, ATM, ATP6V1B2, ATR, ATRX, AXIN2, BAP1, BAP1, BARD1, BLM, BLOC1S3, BLOC1S6, BMPR1A, BRAF, BRCA1, BRCA2, BRIP1, BUB1B, C15orf41, CBL, CCND1, CD70, CDAN1, CDC42, CDC73, CDH1, CDK4, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CEBPA, CEP57, CHEK2, CLCN7, CLPB, COL1A1, COL4A5, COL4A6, CSF2RA, CSF3R, CTC1, CTNNB1, CTSC, CUBN, CXCR4, CYB5R3, CYCS, CYLD, DDB2, DDX41, DHFR, DICER1, DIS3L2, DKC1, DNAJC21, DNASE2, DTNBP1, EFTUD1, EGFR, EGLN1, EGLN2, ELANE, EP300, EPAS1, EPB41, EPB42, EPCAM, EPOR, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6L2, ETV6, EXO1, EXT1, EXT2, EZH2, F10, F11, F12, F13A1, F13B, F2, F5, F7, F8, F9, FADD, FAM111B, FAM172A, FAM175A, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FAP, FAS, FASLG, FBXW7, FGA, FGB, FGFR3, FGFR4, FGG, FH, FH, FLCN, FLCN, FLI1, FLNA, FYB, G6PC3, G6PD, GALNT12, GATA1, GATA2, GBA, GCLC, GFI1, GFI1B, GGCX, GINS1, GP1BA, GP1BB, GP9, GPC3, GPI, GPR143, GPR161, GREM1, GSS, HAX1, HBA1, HBA2, HBB, HFE, HNF1A, HNF1A, HNF1B, HNF1B, HOXA11, HOXB13, HPS1, HPS3, HPS4, HPS5, HPS6, HRAS, IFNGR2, IKBKAP, IKZF1, IL1B, IL1RN, ITGA2, ITGA2B, ITGB3, ITK, JAGN1, JAK2, KIF1B, KIF23, KIT, KITLG, KLF1, KLHDC8B, KRAS, LAMTOR2, LMAN1, LMNA, LPIN2, LYST, LZTR1, MAD2L2, MAGT1, MAP2K1, MAP2K2, MASTL, MAX, MBD4, MC1R, MCFD2, MECOM, MEN1, MET, MITF, MKL1, MLH1, MLH3, MPL, MRE11A, MSH2, MSH3, MSH6, MSH6, MTAP, MTHFD1, MTR, MUTYH, MYH9, MYO5A, NBEAL2, NBN, NF1, NF2, NHP2, NIN, NOP10, NRAS, NSD1, NSUN2, NT5C3A, NTHL1, OCA2, P2RY12, PALB2, PALLD, PARN, PAX3, PAX5, PC, PDGFRA, PDHA1, PDHX, PGM3, PHOX2B, PIEZO1, PIK3CA, PKLR, PMS1, PMS2, PMS2, POLD1, POLE, POLH, POT1, PPM1D, PRF1, PRKACG, PRKAR1A, PROC, PROS1, PTCH1, PTCH2, PTEN, PTPN11, PUS1, RAB27A, RAC2, RAD50, RAD51, RAD51C, RAD51D, RAF1, RASA2, RASAL1, RASGRP2, RB1, RBM8A, RECQL, RECQL4, REN, REST, RET, RFWD3, RHAG, RHBDF2, RINT1, RIT1, RNF139, RNF168, RNF43, RPL11, RPL15, RPL27, RPL31, RPL35A, RPL5, RPS10, RPS19, RPS20, RPS24, RPS26, RPS28, RPS29, RPS7,*

RRAS, RTEL1, RUNX1, SAMD9, SAMD9L, SBDS, SCG5, SDHA, SDHAF2, SDHB, SDHC, SDHD, SEC23B, SERPINC1, SERPINF2, SFTPB, SFTPC, SH2D1A, SHOC2, SLC11A2, SLC19A2, SLC25A38, SLC37A4, SLC45A2, SLC46A1, SLC4A1, SLFN14, SLX4, SMAD4, SMARCA4, SMARCB1, SMARCD2, SMARCE1, SOS1, SOS2, SPRED1, SPTA1, SPTB, SRC, SRGAP1, SRP54, SRP72, STK11, STX11, STXBP2, SUFU, TBXA2R, TCN2, TERC, TERT, TF, THBD, THPO, TINF2, TMEM127, TMPRSS6, TP53, TPI1, TRNT1, TSC1, TSC1, TSC2, TSC2, TUBB1, TYR, TYRP1, UBE2T, UNC13D, USB1, VHL, VHL, VKORC1, VPS13B, VPS45, VWF, WAS, WDR1, WIPF1, WRAP53, WRN, WT1, XAF1, XIAP, XPA, XPC, XRCC2, YARS2,ACTB, ACTG1, BRAF, CBL, EPHB4, HRAS, KAT6B, KRAS, LZTR1, MAP2K1, MAP2K2, MAP3K8, MRAS, NF1, NF2, NRAS, NSUN2, PPP1CB, PTPN11, RAF1, RASA1, RASA2, RIT1, RRAS, SASH1, SHOC2, SMARCB1, SOS1, SOS2, SPRED1, SPRED2, STAMBP. Coverage was 99.8%−100%.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103184.

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