Expanded Genetic Codes in Next Generation Sequencing Enable Decontamination and Mitochondrial Enrichment

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Abstract

We have developed a PCR method, coined Déjà vu PCR, that utilizes six nucleotides in PCR with two methyl specific restriction enzymes that respectively digest these additional nucleotides. Use of this enzyme-and-nucleotide combination enables what we term a “DNA diode”, where DNA can advance in a laboratory in only one direction and cannot feedback into upstream assays. Here we describe aspects of this method that enable consecutive amplification with the introduction of a 5th and 6th base while simultaneously providing methylation dependent mitochondrial DNA enrichment. These additional nucleotides enable a novel DNA decontamination technique that generates ephemeral and easy to decontaminate DNA.

Background

Since DNA sequencing data contains both medical information and patient identification data it presents a unique clinical concern for confidentiality. Many next generation sequencing tests are increasingly making use of universal primers that enable amplification of multiple different patients with the same known primer sequences.

A side effect of utilizing universal primers is that subsequent PCR reaction setups are easily contaminated with PCR products from a previous amplification reaction. A second risk in using universal primers is that it hypothetically affords easier theft of patient medical information as the primers required to amplify a patient contaminant from laboratory equipment or trash are well known (i.e. Illumina Primers). To enable easy destruction of clinical DNA, laboratories have traditionally utilized dUTP in PCR to generate PCR products that are different from genomic DNA and are specifically cleavable with uracil DNA glycosylase (UDG) [1]. Using these methods, only the PCR products that contain uracil are enzymatically digested; therefore, any contaminating PCR products can be digested with no risk of destroying the target DNA about to be amplified. Unfortunately, uracilated DNA is not amplified well with widely-used emulsion or cluster PCR kits, due to the use of uracil-illiterate polymerases in most next generation sequencing platforms [2].

To address this deficit, DREAM PCR replaces this uracil base with the 5th base methylcytosine, as most polymerases are methylcytosine-literate and will efficiently incorporate this base into a PCR product [3]. In addition to 5-methylcytosine ($5me$-dCTP), the recently described “6th base” 5-hydroxymethylcytosine ($5hme$-dCTP) has been the topic of investigation, and many enzymes exist which differentially digest or capture 5-hydroxymethylcytosine [4,5]. Due to its unique biochemical properties, techniques that differentially detect $5hme$C from $5me$C have been the topic of intense focus [6–11], making this an ideal amplification nucleotide to augment DREAM PCR. Both of these methylated nucleotides exist at different frequencies in human genomic DNA [12] and can influence DREAM PCR assay design.

To enable selective serial digestion of the two nucleotides, DREAM PCR substitutes the methyl-specific endonucleases MspJI and AbaSI in place of UDG. MspJI digests heavily methylated PCR products differentially than lightly methylated substrate genomic DNA, and thus it has a preference for digesting double stranded methylated DNA over single stranded lightly methylated circular gDNA presented with a Haloplex exome capture system(Agilent) [3]. This is an important distinction considering the hypermethylated nature of natural CpG islands. Assays targeting CpG islands for sequencing without the single stranded circularization techniques deployed in a Haloplex reaction may choose to use 5-hydroxymethylcytosine as the first amplification nucleotide since its native frequency in gDNA is far lower than the native 5-methylcytosine and thus would better distinguish a contaminant amplicon from a genomic DNA target. For the application of mtDNA sequencing, genomic methyl depletion is preferred due to its concomitant depletion of methylated Nuclear Mitochondrial sequences or NUMTs.

Incorporation of 5-hydroxymethylcytosine enables serial PCR steps to be performed, each with a different 5th base and each respectively digestable with unique enzymes ($5me$CTP+ MspJI and $5hme$-CTP+AbaSI). Such a method offers unique decontamination solutions for more complex massively parallel DNA sequencing workflows requiring more than one amplification step.
Figure 1. DREAM PCR and Déjà vu PCR makes use of what we have termed a “DNA diode” where enzymes that specifically digest 5th and 6th bases respectively are leveraged to ensure complex serial amplification steps can be performed contamination free without physical isolation of lab equipment. Both enzymes are heat inactivated and do not show activity post PCR. Any hmeC products cannot contaminate the Nextera reaction setup as AbaSI is present to selectively digest hmeC-DNA while leaving the target meC DNA intact. Likewise, any Nextera DNA contaminating the LR-PCR setup will be digested by MspJI since it targets both forms of methylation.

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Figure 2. Deleted Mitochondrial DNA hyper-amplifies with LR-PCR. Observed vs Expected coverage of two unique haplogroup mtDNA samples pooled prior to LR-PCR amplification. One 4.5 kb Kearns-Sayre homozygous deleted mtDNA (12.1 kb, KSS mtDNA) sample is mixed with a known wild type mtDNA (16.6 kb, NA12878 mtDNA) sample with a different haplogroup. The KSS mtDNA sample has a unique haplogroup that creates heteroplasmies at expected loci when mixed with a full length mtDNA control. After sequencing the mixtures to 10,000× mean coverage on an Illumina MiSeq V2 system, allele frequencies are measured across a barcoded dilution series where the deleted sample alleles are expected to be seen at 5%, 10%, 15%, 25%, 50%, 75% of the reads. Plotted is the expected coverage of the KSS mtDNA alleles versus the observed ratio (Y-Axis) of the control mtDNA alleles. This is measured by mapping reads with Bowtie and counting allele frequencies at the haplogroup specific loci. This result is expected in that a multiplexed PCR containing 12.1 kb and 16.6 kb molecules will selectively amplify the smaller template. The selective amplification was still observed despite 15 minute extension times applied in PCR. This also highlights the pronounced sensitivity for detecting large deletions in mtDNA samples using LR-PCR.

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Results and Discussion

Consecutive amplification utilizes a 6th base

Several clinically relevant next generation sequencing assays require two serial amplification steps [13,14]. Techniques designed to identify long range genomic phasing often employ whole genome amplification (WGA) before using a more directed PCR approach [15]. In addition, some exome capture techniques require a pre-capture PCR and a post-capture PCR step [16–20]. In applications that require serial PCR, one has to consider which amplification step should include the decontaminating methylated cytosine? We chose to use 16 kb long range PCR (LR-PCR) to amplify the whole mitochondrial genome for subsequent transposon-mediated library construction [21], followed by a secondary 12-cycle amplification step (Nextera PCR reaction) using universal Illumina primers [21].

For serial amplification procedures utilizing universal primers, it would be ideal if two different digestible nucleotides were available for exclusive use in respective amplifications. 5me-dCTP and 5hme-dCTP fit this requirement. Both of these nucleotides are commercially available (Trilink); very recently, the enzyme AbaSI also became available (NEB), and is useful as it selectively digests 5hmeC without digesting 5meC [22]. Both enzymes are heat inactivated and thus remain inactive after the first cycle of PCR.

Decontamination techniques work best when the target to be amplified is different than the product or potential contaminant. If 5me-dCTP exists in the first LR-PCR product, one cannot use MspJI to decontaminate the second Nextera PCR reaction, as MspJI is a methyl-specific restriction enzyme and will digest both the substrate 16 kb target amplicon and any potentially contaminating Nextera PCR products. In order for decontamination to be effective, the post-amplified Nextera contaminants require a nucleotide (here, 5hmeC) that does not exist in the 5meC LR-PCR DNA (Figure 1).

The described LR-PCR has mitochondria specific primers; thus, contaminants from a Nextera PCR reaction with different universal primers are less likely to create amplifiable contamination. Nevertheless, these Nextera libraries contain mitochondrial DNA inserts, a small portion of which is complementary to the LR-PCR primers. This means secondary amplification artifacts can amplify and impair heteroplasmy detection. In addition to this source of background, deleted mitochondria from other clinical samples can hyper-amplify if co-present with clinical full length mtDNA. Figure 2 demonstrates how a patient with a 4.5 kb...
mitochondrial deletion known to be associated with Kearns-Sayre syndrome can hyper-amplify (10X) in a foreground of 16.6 Kb target amplification. These two sources of potential contamination underscore the need for decontamination techniques.

Long range PCR considerations

The use of LR-PCR for massively parallel mitochondrial sequencing has proven to have the most sensitive heteroplasmy and large deletion detection [23–25]. This is largely due to LR-PCR’s ability to deliver uniform coverage and to limit the amplification of similar NUMT sequences [26] found with methods that use hybridization capture techniques. Nevertheless, LR-PCR methods can be hindered by jumping PCR artifacts with NUMTs, meaning that often the heteroplasmy sensitivity is limited to allele frequencies of 1% or greater, despite the fact that sequencing techniques can deliver accurate allele frequencies far below this [26] with other templates. Since 90% of mtDNA deletions are larger than 2 kb, LR-PCR methods are also prone to hyper-amplification of clinically relevant deleted mtDNA samples [27–29].

To address this, we designed a decontamination approach that concurrently depletes methylated NUMTs from the sample. Prior to initiation of PCR, we digest the sample with MspJI as it will digest hyper-methylated dsDNA that can otherwise contaminate the LR-PCR. Exhaustive bisulfite sequencing of mitochondria in several tissues has demonstrated a complete lack of mitochondrial DNA methylation [30], while NUMTs are rapidly methylated in the nuclear genome. This suggests methyl-specific restriction digestion can selectively digest NUMTs and render them non-amplifiable [31,32]. There are two limitations to this application. First, this methyl depletion step utilized in absence of the selectivity of long range PCR may fail to remove non-methylated NUMTs. Secondly, the minor heteroplasmic non-CpG methylation state of mitochondrial control regions in aged or diseased tissue remains a controversial field [33].

During the first LR-PCR amplification we use a mixture of dCTP and 5-me-dCTP. During the second Nextera PCR we use a mixture of dCTP and 5-hme-dCTP. Since MspJI will digest both 5-meC and 5-hmeC, it will decontaminate the LR-PCR reaction setup of both past LR-PCR product and past Nextera PCR product contaminants while also digesting NUMTs gDNA. It is important to note MspJI’s preference of double-stranded DNA over single-stranded DNA and how this preference may alter a given application [34] [35].

After the first LR-PCR and prior to the second Nextera PCR we use AbaSI to digest contaminants as this enzyme only digests 5-hmeC, leaving 5-meC or cytosine intact. In this case, AbaSI will only digest PCR products that contaminate the pre-Nextera sample from the post-secondary PCR process (Figure 3). The second PCR usually contains universal sequencing primers

![Figure 4. DMSO impact on sequencing methylated libraries.](image-url)

Use of DMSO is estimated to lower the Tm 0.6°C per % according to Von Ashen et al. The use of 4%DMSO improves the C1, C10, C20 and C100 sequencing metrics. All samples were deprecated to 400× coverage to normalize read depth. BEDtools was utilized to calculate C1-C100s coverage statistics. The use of 4% DMSO in PCR with 5mCTP improves the C20 coverage of targets in sequencing panels.

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producing small products (700 bp) desired by the limitations of current sequencers. These smaller PCR products can hyper-amplify due to cold PCR or other selective amplification biases and as a result can be over represented. Hyper-amplification of contaminants in PCR a risk in a clinical laboratory testing for heteroplasmy [36].

Decontamination and optimal sequencing performance

Since 5-meC alters the Tm of DNA by 0.5°C per methylated cytosine, optimizations to the PCR conditions are required [37]. Previous studies with DREAM PCR demonstrated decaying sequencing coverage with increasing concentrations of 5-me-dCTP [3]. Raising the annealing and denaturation temperatures to compensate for 5-meC’s impact on Tm exposes DNA to hydrolytic damage [38]. We thus pursued methods that alter the solvation and melting temperature without introducing thermal damage to the DNA. We found that a 3–4% final concentration of DMSO provided optimal sequencing coverage (Figure 4) equal to non-methylated amplification controls.

Of the 354 SNPs identified by GATK (Genome Analysis ToolKit) [39] using the previously published DMSO-free method [3] on NIST (National Institute of Standards and Technology) sample NA12878, 353 variants (99.7% agreement) are found with the 4% DMSO data. The one remaining SNP has evidence of the A>G alternative allele (chr1:116358311) even at a similar allelic ratio (28% vs 31%) but with lower read mapping qualities in the 4% DMSO amplicon. In addition 4% DMSO rescued 7 additional SNPs all present in dbSNP compared to the published methylated SOP. When comparing the 4% DMSO sample to the same control sample run with zero methylation the 4% DMSO provided 358/360 SNPs where the two missing SNPs are C>A and C>T errors (99.4% agreement). This suggests that 4% DMSO in DREAM PCR can compensate for 5meCs known impact on melting temperature.

We measured decontamination by spiking in known amounts of DNA contaminant from a different mitochondrial haplogroup. Then, we treated these samples with the respective enzymes and deeply sequenced (10,000x) to measure the percent heteroplasmy of the sample at the haplogroup specific loci. A simple 1 hr digestion was able to remove equimolar contaminating DNA (Figure 5). This assay is limited in that it is only measuring contamination at 8 haplogroup specific loci.

Mitochondrial enrichment

To measure the mitochondrial DNA enrichment we designed a Haloplex assay that targeted both the entire mitochondrial genome (320 amplicons) and several nuclear genes in parallel (13,060 amplicons). Genomic DNA was purified and treated with and without MspJI digestion (0,0.5, 0.5, 1,2,3 units of MspJI enzyme). We then sequenced these libraries, and mapped the reads to hg19 to measure the ratio of reads mapping to mitochondrial versus nuclear targets. This mapped read ratio is termed the M:N ratio and is used to estimate enrichment. The M:N ratio in the control sample is 12.3 while the MspJI digested...
sample has a M:N ratio of 27.3, demonstrating an enrichment of mitochondrial DNA through the digestion of methylated gDNA. We confirmed the M:N ratio of the source DNA with quantitative PCR (Figure 6 & 7).

Conclusions

Here we report a variation of PCR and sequencing methods incorporating specific enzymatic digestion steps to solve a key reported problem in resolving hydroxymethylcytosine from methylcytosine. Nestor et al. highlighted how challenging this differentiation can be [40] and Wang et al. demonstrated the benefits these enzymes bring to epigenetic studies looking to track the various methylation states with next generation sequencing. Only in recent years has hydroxymethylcytosine been coined the 6th base [8]; this more nuanced view of nucleic acid chemistry raises to question whether the claims of four-nucleotide sequence IDs listed in most gene patents provide sufficient specificity. Many patents also make claims to any complementary sequence of a defined 4 base sequence ID [41]. Complementarity is defined by Chargaff’s rules where the nucleotides base pairing affinity is measured as a function of melting temperature. The use of these expanded nucleotides alters the melting temperature of amplicons significantly in light of Chargaff’s rules. Consider a 25mer oligo with the sequence [CATG]24 with an adenosine as the 25th 3’ prime base. Changing the 3’ base of this oligo to C,G,meC demonstrates a respective shift in Tm of 0.7°C, 0.6°C, 0.2°C, 1.1°C (IDT oligo design tools). This dramatic shift in Tm shown by 5-meC suggests complementarity claims are challenged with the use of 5-me-dCTP in PCR. It is also unclear how Hoogstein base pairing will be interpreted regarding complementarity patent language and if the use of 7-deaza dGTP challenges such claim language since this non-natural nucleotide also alters melting temperature and Hoogstein pairings [41].

Additionally, expanded genetic codes in target amplification can provide both additional error correction opportunities [42,43] in DNA sequencing and valuable decontamination tools. Since these bases randomly incorporate into GC-rich regions and AbaSI and MspII cut distal to the methylated base, they can be utilized as a targeting tool for directed fragmentation of recalcitrant GC-rich templates and offer valuable tools for gap closure similar to those methods described by McMurray et al [44].

These results demonstrate additional utility of DREAM PCR in decontaminating more complex amplification procedures than described previously [3]. In addition we underscore the importance of such decontamination techniques for mitochondrial sequencing and the impact of suppressing large deletion hyperamplification. We also demonstrate a beneficial enrichment of mtDNA by leveraging the lack of methylation in mitochondrial DNA. This addresses a problem with NUMTs contaminating many next-generation mitochondrial sequencing assays previously described and may open the field for accurate sub percentage heteroplasmy sensitivity.

These results likely have relevance for accurate sequencing in any sample that demands low allele frequency quantification like heterogeneous biopsies. Likewise, the results underscore the value in generating ephemeral PCR products. With recent concerns over DNA confidentiality and the ease of de-identification of DNA samples [45], data encryption is becoming a standard in clinical laboratory data management to prevent in-silico contamination or disclosure of DNA sequence [46,47]. Considering physical DNA can be harvested from 50,000 year old samples [48], a clinical laboratory’s trash is a confidentiality exposure point if DNA is not digested or destroyed during testing. Thus methods that eliminate...
DNA from a clinical laboratory offer attractive and responsible features. In summary, we demonstrate a method that improves DREAM PCR sequencing performance while concurrently providing a more responsible clinical management of patient DNA.

**Materials and Methods**

All data for this project has been submitted to the European Nucleotide Archive, http://www.ebi.ac.uk/ena/data/view/PRJEB5732.

**Long-range PCR**

PCR setup utilized forward and reverse primers for the ~16 kb product: mtPCR6F-321-5'TGGCCACAGCACTTAAACACATCTC 3' and mtPCR6R-16191-5'TGCTGTACTTGCTTGTAAGCATGGG3'. 699 bases are omitted from the D-LOOP due to positive amplification being obtained using those sequences with Rho negative cells (cells with no mitochondria). PCR was performed utilizing 50 ng of gDNA (10 ng/ul). Reaction setup included 1.5 ul of DNA, 5.0 ul of 10 X LA PCR Buffer II, 0.5 ul TaKaRa LA Taq DNA polymerase, 10.65 ul ddH20, and 0.125 ul (50 uM) of each primer with 8.0 ul dNTP mixture (2.5 mM each dNTP where a ratio of 87.5:12.5 dCTP:5me-dCTP). The 50 ul PCR reaction was cycled with an initial 1 minute denaturation at 94°C and is followed by 30 cycles of 98°C at 10 s, 68°C for 15 minutes. A final 72°C 10 minute extension is performed prior to 4°C hold. PCR products are purified using 75 ul of Ampure (Beckman Genomics).

**Nextera reaction and 5-hydroxymethylcytosine PCR**

3 ul (2.5 ng/ul) of the purified LR-PCR product is used in a 10 ul Nextera reaction (1/20thX) utilizing 5.0 ul TD, 0.25 ul of TDE, 1.75 ul ddH20 (acronyms according to manufacturers instructions). Samples are incubated for 30 minutes at 55°C followed by a 15 ul Ampure purification. Products are eluted in 25 ul of ddH20 and 10 ul of eluent are used for Nextera PCR with 0.75 ul of each 10 uM primer, 1.25 ul of each Illumina index, 20 ul of 2× Q5 polymerase (New England Biolabs) and 0.75 ul of 5 mM 5-hydroxymethylcytosine (Trilink) with a 4% final DMSO. 12 Cycles of PCR are performed with the following cycling protocol: 72°C for 3 minutes, 98°C for 30 seconds, 12 cycles of 98°C for 10 seconds, 63°C for 30 seconds, 72°C for 1 minute. PCR products are purified using 52.5 ul of Ampure. These products are optionally size selected with a SAGE Sciences Pippin PrepII system in the 600–800 bp size range for 2×250 bp sequencing on a MiSeq V2 sequencer from Illumina according to the manufacturers instructions.

**Decontamination**

MspJI digestion is performed with 100 ng DNA, 1 X buffer, 1 X Activator, 1 X BSA, 0.07 U MspJI at 37°C for 30 minutes. The sample is heat killed at 65°C for 20 minutes before initiating PCR. AbaSI digestion is performed with 1 ng DNA, 1 X buffer, 0.3 U AbaSI, at 25°C for 2 hours. The sample is heat killed at 65°C for

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**Figure 7. Confirmation of mtDNA copy number with qPCR.** SYBR Green Real Time PCR of mtDNA genes ND1 and ND6 estimates mitochondrial copy number at 428 copies next to diploid genes BECN1 and NEB.

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20 minutes before initiating PCR. Figure 3 demonstrates the decontamination with AbaSI with quantitative PCR.

**Enrichment ascertaining**

Haloplex assays were designed and amplified according to the manufacturers version 2 instructions (Agilent). MspJI digestion was performed as described above but with various concentrations of enzyme. Experiments were DNA barcoded and sequenced with Illumina MiSeq V2 sequencer with 2x250 bp reads to ensure high mapping quality. All reads were mapped with Bowtie2 and coverage calculations were performed with BEDTools as previously described [5].

The control samples demonstrated a M:N ratio of 12.3. This is very close to theoretical expectations as the size of the amplicon BED file for the mitochondrial and nuclear targets is larger than the desired targets to be sequenced and this presents a M:N amplicon target ratio of 64.8 kb/2.7 Mb and or expected M:N ratio of 0.0236 assuming equimolar copy number. Quantitative PCR suggests a mitochondrial copy number of 428 relative to nuclear control genes. The copy number adjusted M:N is 10 (0.0236*428) and represents the expected M:N ratio we should see in sequencing according to qPCR estimates of the mtDNA in consideration of the in-silico amplicon design. The M:N ratio of the 3 units of MspJI treated gDNA samples is over twice as high (27.3) as the controls (Figure 6). To further confirm these results we used magnetic particles (New England Biolabs, EpiMark) with Methyl Binding Domain (MBD) to methyl capture and sequence a given sample to demonstrate far lower M:N ratios. The MBD particles deliver confirmatory evidence for differential methylation between Mitochondrial and Nuclear DNA (Figure 6).

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**Author Contributions**

Conceived and designed the experiments: KJM RB. Performed the experiments: JS LZ. Analyzed the data: SM JW VT. Contributed reagents/materials/analysis tools: AZ RB. Wrote the paper: KJM.

**References**

1. Longo MC, Berninger MS, Hartley JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 93: 125–128.
2. Warde J, Burgers PM, Cahn IK, Darley K, Heslop P, et al. (2008) Uracil recognition by replicative DNA polymerases is limited to the archaea, not occurring with bacteria and eukarya. Nucleic Acids Res 36: 705–711.
3. McKerman KJ, Spangler J, Hellert Y, Zhang L, Tadigotla V (2013) DRAMing of a patent-free human genome for clinical sequencing. Nat Biotechnol 31: 984–987.
4. Horton JR, Malbush MY, Cohen-Karni D, Zhang X, Griggs RM, et al. (2012) Structure and cleavage activity of the tetrameric MspJI DNA modification-dependent restriction endonuclease. Nucleic Acids Res 40: 9763–9773.
5. Cohen-Karni D, Xu D, Aponte L, Fomenvok A, Sun Z, et al. (2011) The MspJI family of modification-dependent restriction endonucleases for epigenetic studies. Proc Natl Acad Sci U S A 108: 11040–11045.
6. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324: 929–932.
7. Kraus TF, Globisch D, Wagner M, Eigenbrod S, Wiedmann D, et al. (2012) Low values of 5-hydroxymethylcytosine (5hmC), the “sixth base,” are associated with anaplasia in human brain tumors. Int J Cancer 131: 1577–1590.
8. Munzel M, Globisch D, Bruckl T, Wagner M, Welmüller V, et al. (2010) Quantification of the sixth DNA base 5-hydroxymethylcytosine in the brain. Angew Chem Int Ed Engl 49: 5375–5377.
9. Munzel M, Globisch D, Carell T (2011) 5-Hydroxymethylcytosine, the sixth base of the genome. Angew Chem Int Ed Engl 50: 6460–6468.
10. Jin NG, Wu, Nipher GP (2011) Genomic mapping of 5-hydroxymethylcytosine in the human brain. Nucleic Acids Res 39: 5013–5024.
11. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, et al. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL parter TET1. Science 324: 930–935.
12. Lee K, Hamn J, Whitworth K, Sapse L, Park KW, et al. (2014) Dynamics of TET family expression in porcine preimplantation embryos is related to zygotic genome activation and required for the maintenance of NAONO. Dev Biol 386: 86–95.
13. Igartua C, Turner EH, Ng SB, Hodges E, Hannon GJ, et al. (2010) Targeted enrichment of specific regions in the human genome by array hybridization. Curr Protoc Hum Genet Chapter 16: Unit 16.13.
14. Ng SB, Turner EH, Robertson PD, Flygare SD, Bigham AW, et al. (2009) Targeted capture and massively parallel sequencing of 12 human exomes. Nature 461: 272–276.
15. Peters BA, Kermogi BM, Sparks AB, Allerow O, Heng P, et al. (2012) Accurate whole-genome sequencing and haplotyping from 10 to 20 human cells. Nature 487: 190–195.
16. Gilissen C, Arts HH, Hoischen A, Spruijt L, Mans DA, et al. (2010) Exome sequencing identifies WDR35 variants involved in Sensenbrenner syndrome. Am J Hum Genet 87: 418–423.
17. Gilissen C, Hoischen A, Brunner HG, Velman JA (2011) Unlocking Mendelian disease using exome sequencing. Genome Biol 12: 228.
18. Gilissen C, Hoischen A, Brunner HG, Velman JA (2012) Disease gene identification strategies for exome sequencing. Eur J Hum Genet 20: 490–497.
19. Haack TB, Danhauser K, Habererger B, Hoser J, Streckier V, et al. (2010) Exome sequencing identifies ACAD9 mutations as a cause of complex I deficiency. Nat Genet 42: 1131–1134.
20. Klassen T, Davis C, Goldman A, Burgess D, Chen T, et al. (2011) Exome sequencing of ion channel genes reveals complex profiles confounding personal risk assessment in epilepsy. Cell 145: 1036–1048.
21. Tarnopolsky M, Meaney B, Robinson B, Sheldon K, Boys RG (2013) Severe infantile Leigh syndrome associated with a rare mitochondrial ND6 mutation, m.14487T>C. Am J Med Genet A 161: 2029–2035.
22. Wang H, Guan S, Quinby A, Cohen-Karni D, Pradhan S, et al. (2011) Comparative characterization of the PvuRtsI family of restriction enzymes and their application in mapping genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39: 9294–9305.
23. Zhang W, Cui H, Wong IL (2012) Comprehensive one-step molecular analyses of mitochondrial genome by massively parallel sequencing. Clin Chem 58: 1322–1331.
24. Cui H, Li F, Chen D, Wang G, Tsuang CK, et al. Comprehensive next-generation sequence analyses of the entire mitochondrial genome reveal new insights into the molecular diagnosis of mitochondrial DNA disorders. Genet Med.
25. Fulk M, Pierce EA, Conougar M, Xia MH, Guadalupe M, et al. (2012) Mitochondrial disease genetic diagnostics: optimized whole-exome analysis for all MitoCarta nuclear genes and the mitochondrial genome. Discov Med 14: 388–399.
26. Li M, Schroeder R, Ko A, Stonerking M (2012) Fidelity of capture-embouchure for mtDNA genome sequencing: influence of NUMTs. Nucleic Acids Res 40: e137.
27. Danas J, Carneiro J, Goncalves J, Stewart JB, Samuels DC, et al. (2012) Mitochondrial DNA deletions are associated with non-B DNA conformations. Nucleic Acids Res 40: 7606–7621.
28. Smit A, Rizzuto R, Moraes CT, Banske S, Arnaudo E, et al. (1990) Recombination via flanking direct repeats is a major cause of large-scale deletions of human mitochondrial DNA. Nucleic Acids Res 18: 561–567.
29. Kreuder J, Repp R, Borkhardt A, Lampert F (1995) Rapid detection of mitochondrial deletions by long-distance polymerase chain reaction. Eur J Pediatr 154: 996.
30. Hong EE, Okinaka GM, Smith AD, Hsieh CL (2013) Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methyla- tion in human mitochondrial DNA. Mol Cell Biol 33: 2603–2609.
31. Keller I, Benassam D, Nichols RA (2003) Transition/transversion bias is not universal: a counter example from grasshopper pseudogenes. PLoS Genet 3: e22.
32. Haakani-Covo E, Zeller RM, Martin W (2010) Molecular poltergeists: mitochondrial DNA copies (numts) in sequenced nuclear genomes. PLoS Genet 6: e1000934.
33. Bellizi D, D’Aquila P, Scafone T, Giordano M, Riso V, et al. (2013) The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. DNA Res 20: 537–547.
34. Lay MJ, Wittwer CT (1997) Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clin Chem 43: 2262–2267.
35. Riise KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysing DNA melting curves during the polymerase chain reaction. Anal Biochem 246: 134–160.
36. Li J, Wang L, Mannon H, Kulke MH, Berrecio B, et al. (2008) Replacing PCR with COLD-PCR enriches variant DNA sequences and reduces the sensitivity of genetic testing. Nat Med 14: 579–581.
37. von Ahnen N, Wittwer GT, Schutz E (2001) Oligonucleotide melting temperatures under PCR conditions: nearest-neighbor corrections for Mg(2+), deoxynucleotide triphosphate, and dimethyl sulfoxide concentrations with comparison to alternative empirical formulas. Clin Chem 47: 1956–1961.

38. Peak MJ, Robb PT, Peak JG (1995) Extreme resistance to thermally induced DNA backbone breaks in the hyperthermophilic archaeon Pyrococcus furiosus. J Bacteriol 177: 6316–6318.

39. McKenna A, Hanna M, Banks E, Siyachenko A, Cibulskis K, et al. (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20: 1297–1303.

40. Nestor C, Razov A, Mecham R, Duncan D (2010) Enzymatic approaches and bisulfite sequencing cannot distinguish between 5-methylcytosine and 5-hydroxymethylcytosine in DNA. Biotechniques 48: 317–319.

41. Holman CM (2012) Debunking the myth that whole-genome sequencing infringes thousands of gene patents. Nat Biotechnol 30: 240–244.

42. Keith JM, Adams P, Bryant D, Cochran DA, Lala GH, et al. (2004) Unlocking hidden genomic sequence. Nucleic Acids Res 32: e35.

43. McMurray AA, Sulton JE, Quail MA (1998) Short-insert libraries as a method of problem solving in genome sequencing. Genome Res 8: 562–566.

44. Homer N, Srininger S, Redman M, Duggan D, Tembe W, et al. (2008) Resolving individuals contributing trace amounts of DNA to highly complex mixtures using high-density SNP genotyping microarrays. PLoS Genet 4: e1000167.

45. Trakalis YJ (2012) Patient-controlled encrypted genomic data: an approach to advance clinical genomics. BMC Med Genomics 5: 31.

46. Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, et al. (2012) Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 30: 1033–1036.

47. Lalueza-Fox C, Gilbert MT (2011) Paleogenomics of archaic hominins. Curr Biol 21: R1002–1009.