

Isothermal multiple displacement amplification: a methodical approach enhancing molecular routine diagnostics of microcarcinomas and small biopsies

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Background and methods: Isothermal multiple displacement amplification (IMDA) can be a powerful tool in molecular routine diagnostics for homogeneous and sequence-independent whole-genome amplification of notably small tumor samples, eg, microcarcinomas and biopsies containing a small amount of tumor. Currently, this method is not well established in pathology laboratories. We designed a study to confirm the feasibility and convenience of this method for routine diagnostics with formalin-fixed, paraffin-embedded samples prepared by laser-capture microdissection.

Results: A total of 250 µg DNA (concentration 5 µg/µL) was generated by amplification over a period of 8 hours with a material input of approximately 25 cells, approximately equivalent to 175 pg of genomic DNA. In the generated DNA, a representation of all chromosomes could be shown and the presence of elected genes relevant for diagnosis in clinical samples could be proven. Mutational analysis of clinical samples could be performed without any difficulty and showed concordance with earlier diagnostic findings.

Conclusion: We established the feasibility and convenience of IMDA for routine diagnostics. We also showed that small amounts of DNA, which were not analyzable with current molecular methods, could be sufficient for a wide field of applications in molecular routine diagnostics when they are preamplified with IMDA.

Keywords: isothermal multiple displacement amplification, isothermal, whole-genome amplification, routine diagnostics, biopsies, microcarcinomas, microdissection

Introduction

The modern molecular diagnostic field is faced with the problem of an increasing spectrum of analysis from decreasing amounts of tissue, providing the opportunity for “tailored” therapy beneficial for the patient. The current approaches focus on collecting more material for the preferred molecular analysis, eg, by using cryobiopsies for evaluation of lymph node tissue in lung cancer.¹⁻³ With respect to the patients health, a better way would be to improve the preamplification methods already in use to accumulate an adequate amount of DNA/RNA for all types of analysis required. Although many different types of preamplification methods have been published and evaluated, none of them have been accepted for routine diagnostics.⁴⁻¹⁷

In our opinion, a promising and powerful DNA preamplification method for this application is isothermal multiple displacement amplification (IMDA). This is an isothermal preamplification method that combines the advantages of non-thermostable DNA polymerases with a high yield reaction.^{10,11,14,18-21} Whole-genome amplification methods (WGA) using thermostable DNA polymerases may often

generate non-specific amplification artifacts, resulting in an incomplete coverage of loci and/or generating very short DNA fragments.¹⁸ Compared with other whole-genome amplification methods, IMDA, reported for the first time by Dean et al in 2002,¹⁸ shows a highly uniform coverage across the genome and generates high molecular weight genomic DNA (up to >70 kb in length). This method is already validated for clinical specimens^{10,11} as well as for formalin-fixed paraffin-embedded (FFPE) tissue.^{5,10} In microbiology, IMDA has become state of the art as a pre-polymerase chain reaction (PCR) method to amplify difficult samples and low copy number sequences.²² It is sufficient to start with an input of only a few laser-capture microdissected cells for whole-genome amplification to generate an adequate amount of DNA for further molecular analysis.

The aim of this study was to confirm the convenience and feasibility of IMDA for routine molecular diagnostics using very small tumor samples, including small biopsies with a low number of malignant cells or microcarcinomas.

Materials and methods

Study design

To confirm the feasibility of IMDA for routine molecular pathological diagnostics, different FFPE lung tissue samples were microdissected and subsequently preamplified by IMDA. For pretesting of amplification efficiency, only benign tissue was used. To test the homogeneity of the amplification at different chromosomal loci, 33 targets distributed all over the human genome (at least one on every chromosome) were tested by SYBR-green (Life Technologies, Carlsbad, CA, USA) quantitative PCR analysis. Afterwards, human lung adenocarcinoma samples were resequenced after initial DNA preamplification using IMDA for known *EGFR* exon 21 c.2573T>G (p.L858R) point mutations by Sanger sequencing. Well known activating *EGFR* exon 19 deletions were determined using agarose gel electrophoresis. The known *EGFR* exon 19 deletions can be clearly separated by this method. In addition, the same tumor samples were sequenced by pyrosequencing for possible *ERCC1* exon 4 c.19007T>C (p.N118N) and 3'-UTR c.8092C>A polymorphisms, respectively.

Samples and patients

Only FFPE tissue was used. FFPE preparation was performed according to the institutional standards. The fresh tissue was fixed in 4% buffered formalin for 24 hours, then diced to approximately 0.5 × 1.0 × 1.0 cm dimensions and embedded in paraffin. For diagnostic classification, one to four,

<1 μm thick sections were used for immunohistochemistry and staining with hematoxylin and eosin.

For evaluation of IMDA, ten benign lung tissue samples were selected for pretesting. Additionally, for sequence analysis, four lung cancer specimens were selected for evaluation. One sample contained a known deletion in exon 19 of the *EGFR* gene and another sample contained a known p.L858R point mutation in exon 21 of the *EGFR* gene. The remaining two tumor samples included in the analysis show a wild-type *EGFR* gene locus. Cells for subsequent IMDA were separated from inflammation and leukocytes by microdissection.

Microdissection

Sample slides were microdissected using laser-capture microdissection technology (PALM RoboMover; Zeiss, Jena, Germany). Zeiss MembraneSlide NF 1.0 PEN glass slides were used due to the plastic film affixed on the slides. The Robo-LPC computer program was used to assist the microdissection procedure.

For tissue lysis prior to IMDA amplification, Qiagen FFPE lysis solution (Qiagen, Hilden, Germany), which is applied in the REPLI-g FFPE kit (Qiagen), was used; 20 μL was directly dropped into the cap of the microdissection tube. The microdissected tumor areas had dimensions in the range of 50 × 50 μm to 150 × 100 μm correlating with an average of approximately 25 tumor cell nuclei.

IMDA preamplification

The commercial REPLI-g FFPE kit (Qiagen) was used for IMDA. This assay combines a ligase reaction to generate high molecular weight DNA templates from degraded FFPE tissue with IMDA DNA preamplification. Genomic DNA from the lysed tissue was denatured at room temperature for 3 minutes and then incubated with random primers. Strand displacement synthesis was performed at 30°C according to the manufacturer's recommendations. As the amplification reaction reaches a plateau after approximately 8 hours, the reaction was terminated by incubation at 60°C for 10 minutes.

DNA quantification was done using a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA).

Amplification products were stored at 4°C until further use.

Analysis of chromosomal coverage

To test for the presence of all chromosomes/target sequences in the amplified DNA, randomly chosen genes spanning regions on every chromosome were quantitatively tested

using Platinum SYBR Green quantitative PCR SuperMix-UDG (Invitrogen). In addition, on four chromosomes, multiple gene loci were amplified to test the relative representation of different regions on these chromosomes. Quantitative analysis was performed on a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta C_t}$ method was used to normalize target C_t 's versus reference C_t 's. To ensure amplification of the probably highly degraded genomic DNA from the FFPE tissue, amplicons for quantitative PCR reaction were designed with a maximum length of 100 bp. All primer sequences are listed in Table 1, and the PCR protocol is shown in Table 2.

Mutational analysis

Sanger sequencing was performed on an Applied Biosystems 3500 genetic analyzer according to the manufacturer's protocol (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems) with a halved reaction volume. Pyrosequencing was performed on a PyroMark Q24 (Qiagen). For data analysis, the Qiagen PyroMark Q24 software was used.

Sequencing was done according to the Qiagen PyroMark Q24 protocol. Streptavidin sepharose (GE Healthcare, Chalfont, UK) was used instead of streptavidin-coated beads. Sequences of all sequencing primers are listed in Table 3.

Results

IMDA preamplification

IMDA was successful in all (malignant and benign) samples. With an input of approximately 20–25 cells (equaling approximately 175 pg of genomic DNA) for laser-capture microdissection, 3 $\mu\text{g}/\mu\text{L}$ up to more than 5 $\mu\text{g}/\mu\text{L}$ DNA was generated with a reaction volume of 50 μL . The actual amount of suitable DNA amplified strongly depends on the duration of the final amplification step.

The best results were obtained with an amplification duration of 8 hours. At this point, all quantitative PCR analyses showed sufficient results; nevertheless the amplification efficiency showed small disparities. The age of the tissue slides, did not show an influence on the quality of the quantitative PCR template. All negative controls showed no results.

Table 1 List of all primer sequences used for amplification for relative chromosomal representation analysis

Gene name	Chromosome	Forward	Reverse
<i>MTOR</i>	1	AAGAGTTCCTGTTTCATGTGG	CAGGTGGTAGCTCTGGCTTC
<i>CASP8</i>	2	TCAGAGACCTGCACTTGTGG	AACAACCCAGCAAAGGTGTC
<i>AGTR1</i>	3	ACCTGCTCCTGGTAGAGCAA	CAGCCACACTCTGAGGTGAA
<i>VEGFR2</i>	4	CCGAGTGGGCACAGTTTATT	ACAATTCACACTGCCATCCA
<i>IL-4</i>	5	CAGTTGGAGGGAGTGAGAGC	TGCACAGAGTCTTGGTCCAG
<i>IL-13</i>	5	TGCACGTGCTTTATGTGTCA	GCTTGAATCCAGCCTCTGTG
<i>HLA-A</i>	6	ATTACATCGCCCTGAACGAG	CCAGGTAGGCTCTCAACTGC
<i>EGFR</i>	7	GTACTCACTGTGCGCCCAT	TTCCAAGACGCTTCTGACCT
<i>FGFR1</i>	8	GAACAGGCATGCAAGTGAGA	GCTGTAGCCCTGAGGACAAG
<i>CTSB</i>	8	TCTCGGTCTCAGCCAGTTCT	CCAGTAGGGTGTGCCACTCT
<i>ANGPT2</i>	8	TCATTTGACGCATTGACCCTA	GCTCCAAGATCCGTTCTGAG
<i>JAK2</i>	9	CTGCCTTGTGACTGACTGGA	AGCCTACCTGGGCTATCCAT
<i>VIM</i>	10	GGCCAGCTGTAAGTTGGTA	CCTAGCGGTTTAGGGGAAAC
<i>HBB</i>	11	TTGGACCCAGAGTTCTTTG	CACTAAAGGCACCGAGCACT
<i>GAPDH</i>	12	CGACCACTTTGTCAAGCTCA	AGAGTTGTGAGGGCCCTTTT
<i>ERCC5</i>	13	AGCCAGTAGCAGGAAGTGA	GAAAACCCTCCAAAGCTTCC
<i>FOXO1</i>	13	CACTGTATGCTCTGGCAGGA	GCAGTCAGGGCTGAGAAATC
<i>ERS2</i>	14	CTACCAGCAGCCAGCTATC	GTCTTTATGGCCACCCTGAA
<i>CYP1A2</i>	15	TAATCCCCAGCTCTGGTGTG	CATTTTATGCCCATTTGACC
<i>HBA1</i>	16	CCACTGACCCTCTTCTCTGC	GAAGCCAGGAACCTGTCCAG
<i>p53</i>	17	TGTCATCTCTCCTCCCTGCT	TCTGAGTCAGGCCCTTCTGT
<i>BCL2</i>	18	GCTTATCGTCTGCGTGTGAA	GGGATGTGCTCGGTAAAGTGT
<i>TYMS</i>	18	TCTGGAAGGGTGTGTTGGAG	AGGATCTCACTGGCTGCTGT
<i>ERCC1</i>	19	AAGGCACTGTCCATTCATCC	TTGAGCTAGGGAGGGTAGCA
<i>TOP1</i>	20	TTGGCTGTTGCAGATAGTC	CACAGAAATGCCACATGACC
<i>ERG</i>	21	TTCCCTGGGTCTGAAAGATG	ATTGTGTTCTGCGGGTTAGG
<i>MAPK1</i>	22	CTCAGCCTTACCCTTTGCTG	TGTGGTGTGAGGGGTACAGA
<i>AR</i>	X	GGCTGACATACATGGGCTTT	CTGCAATGACCTCACGCTTA
<i>TSPY1</i>	Y	TCGGGGAAGTGTAAGTGACC	CAATAGTACCCACGCCTGCT

Note: All generated amplicons are about 100 bp in length.

Table 2 Details regarding the PCR conditions

Reagent mix		Thermocycling program			Cycles
Reagent	μL	Step	Temperature, $^{\circ}\text{C}$	Time (hh:mm:ss)	
PCR buffer (10 \times)	5.0	1	95	00:10:00	1
MgCl ₂	5.0	2	95	00:00:30	40
Primer forward (10 μM)	1.0		55	00:00:30	
Primer reverse (10 μM)	1.0		72	00:01:00	
dNTPs	1.0	3	8	∞	1
TaqPol	0.5				
H ₂ O	35.5				
Template	1.0				
Total	50.0				

Notes: The protocol was used for all performed PCR reactions including quantitative PCR for chromosomal coverage determination as well as PCR for subsequent sequencing procedures. Time in the thermocycling program is expressed in hours, minutes, and seconds.

Abbreviations: dNTPs, deoxynucleotide triphosphates; PCR, polymerase chain reaction; hh, hours; mm, minutes; ss, seconds.

Chromosomal coverage

All tested genes on all chromosomes could be detected. However, the detected gene loci differed in their amplification (Figure 1). Both the chi-squared test and Fisher's Exact test showed a significant independency for amplification (P equals nearly 1). Also, within the chromosomes, there were clear differences (Figure 1). Differences in amplification efficiency between the analyzed gene loci ranged up to 3.8-fold between the median $2^{-\Delta\text{Ct}}$ values of each locus.

The amplified sequence seems not to have a major impact on this phenomenon; it rather depends on the quality of DNA in the tissue slides being investigated than on the parameters of the target sequence. Most of the amplified DNA fragments were of a small size. This effect becomes increased by the ligation step, which links together several fragments of distinct genes/regions. Small-sized DNA fragments like these "patchwork fragments" would still be long enough for next-generation sequencing, and the merging of different

gene sequences is not problematic, but also for normal "qualitative" analysis, like mutational analysis, the amount of useful amplified DNA is adequate.

Mutational analysis

After an initial IMDA preamplification, two specimens were tested for known *EGFR* mutations (exon 19 deletions and exon 21 p.L858R point mutations) by capillary sequencing. Both mutations could be detected reproducibly in all replications (Figure 2). In two patient samples that contained the *EGFR* wild-type sequence and served as negative controls, no mutations could be detected after IMDA preamplification.

The same four specimens were used for pyrosequencing of *ERCC1* point mutations at the c.19007T>C and c.8092C>A mutation hotspot of *ERCC1*. Sequencing was successful in all four cases. One case showed an *ERCC1* exon 4 c.19007T>C, whereas the others were classified as wild-type (Figure 3). For *ERCC1* c.8092C>A mutation status,

Table 3 List of primers used for pyrosequencing and amplification

Method	Gene name	Direction	Sequence
Sanger sequencing	<i>EGFR</i> exon 19	Forward	TATGTA AAAACGACGGCCAGTTGGCACCATCTCACAATTGC
	<i>EGFR</i> exon 19	Reverse	TATTATAGGGCGAATTGGGTGCTGCCAGACATGAGAAAAGG
	<i>EGFR</i> exon 21	Forward	TATGTA AAAACGACGGCCAGTA ACTACTTGGAGGACCGTCGC
	<i>EGFR</i> exon 21	Reverse	TATTATAGGGCGAATTGGGTTCCTTACTTTGCCTCCTTCTGC
	<i>EGFR</i> exon 21	Sequencing (F)	GTAAAACGACGGCCAGT
	<i>EGFR</i> exon 21	Sequencing (R)	TATAGGGCGAATTGGGT
Pyrosequencing	<i>ERCC1</i> codon 118	Forward	BIO -TCCCTATTGATGGCTTCTGC
	<i>ERCC1</i> codon 118	Reverse	TCCAGA AACTGGGACATGA
	<i>ERCC1</i> codon 118	Sequencing	ACGTCGCCAAATTCCCAGGG
	<i>ERCC1</i> C8092A	Forward	TGAGCCAATTCAGCCACT
	<i>ERCC1</i> C8092A	Reverse	BIO -TAGTTCCTCAGTTTCCCG
	<i>ERCC1</i> C8092A	Sequencing	AGGCCGGGACAAGAAGCGGA

Note: One of the amplification primers always has a biotin (**BIO**) tag for purification and linkage to the streptavidin-coated beads.

Abbreviations: Sequencing (F), forward primer used for sequencing; Sequencing (R), reverse primer used for sequencing.

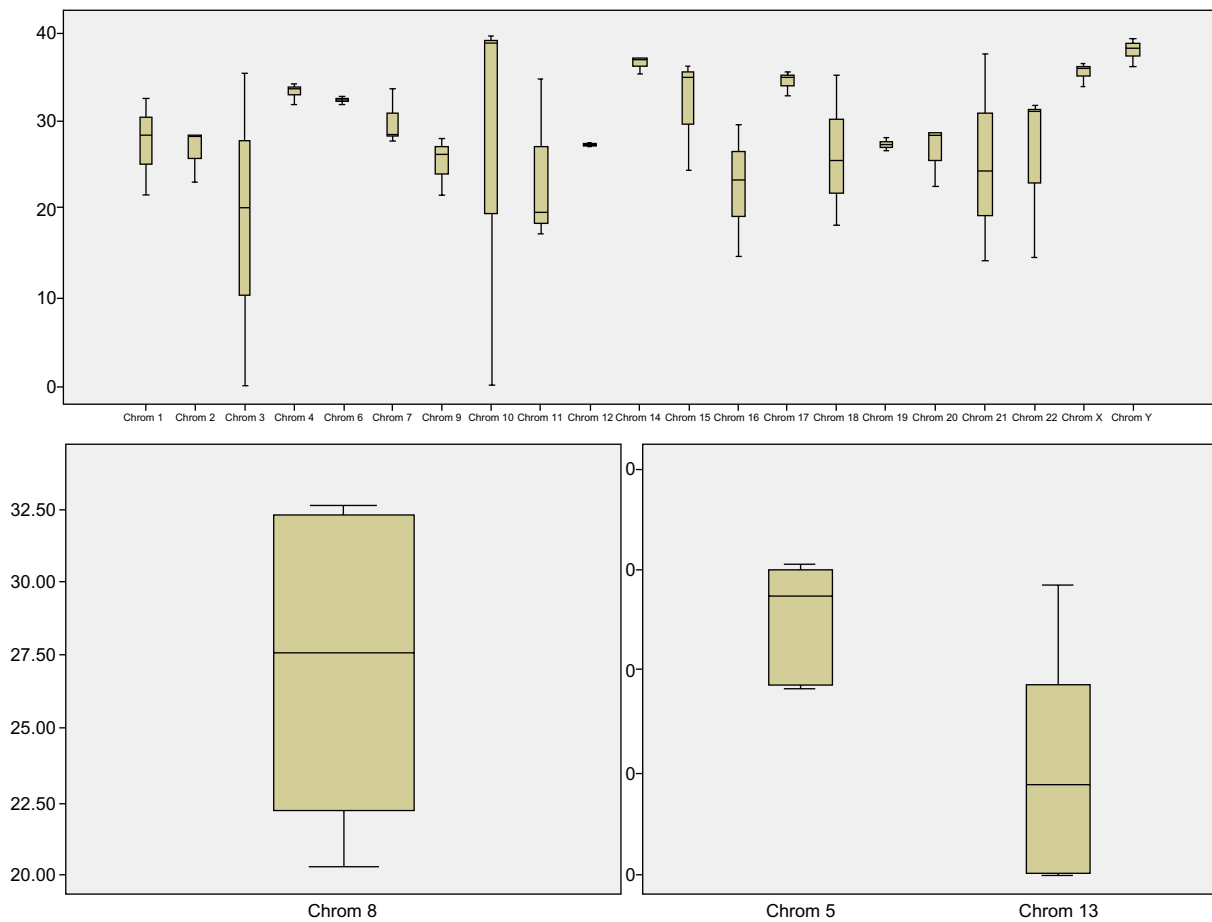


Figure 1 Amplification strength of tested genes normalized to *GAPDH* and displayed by $2^{-\Delta\Delta C_t}$ values. Six genes show an extraordinary deviation (*VIM*, *FOXO1*, *HBA1*, *AR*, *MAPK1*, *TSPY1*), but most of them have a lower amplification rate (except *TSPY1*). The variances can be explained by the difference in amplification product length and the quality of the template before ligation.

Abbreviation: Chrom, chromosome.

all cases showed a wild-type genotype in comparison with a mutated positive control.

Discussion

The aim of our study was to check the feasibility and convenience of IMDA as an isothermal preamplification method

for molecular diagnostics involving notably small sample material.

Our results show outstanding amplification efficiency (250 μg of genomic DNA for approximately 25 cells) and that IMDA is a valuable preamplification method for all further molecular analysis of these clinical specimens.

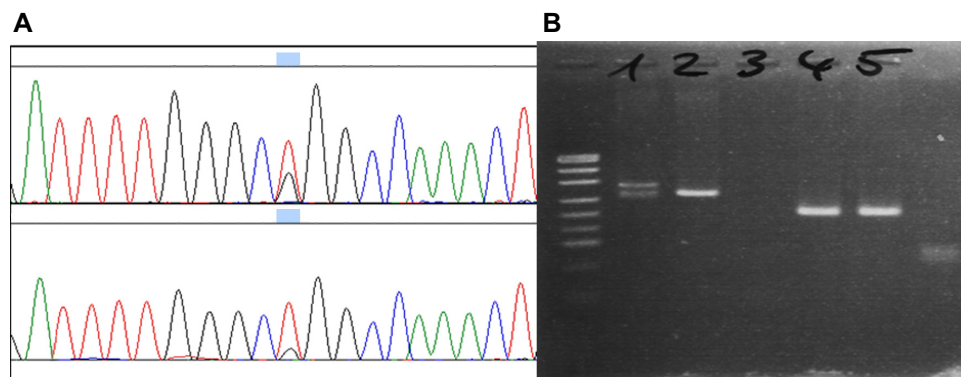


Figure 2 Chromatograms of Sanger sequencing for *EGFR* mutations in clinical specimens (A) exon 21 p.L858R wild mutated sample (B) exon 19 deletion in the agarose gel (1) compared with an exon 19 wild-type (2), a non-template control (3), as well as amplicons for *EGFR* exon 21 sequencing (4 and 5).

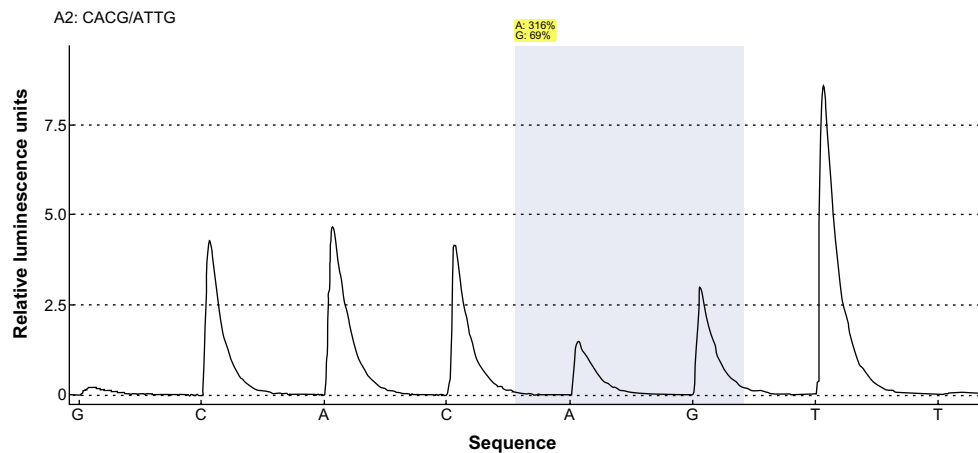


Figure 3 Pyrosequencing flowgram showing an *ERCC1* exon 4 c.19007T>C heterogeneous genotype (p.N118N). The first injected base (G) was used to identify the background, and the last injected base (T) was used for determination of phasing effects. The shaded area marks the position analyzed for possible variants.

In contrast, recent studies show a five- to ten-fold lower efficiency of IMDA, but these deviations may be based on differences between the quantification methods used.¹⁸ The additional preceding ligation step to generate DNA templates of higher genomic weight could also be a possible explanation for this phenomenon.

All 33 investigated genes could be amplified and detected by subsequent analysis, although the amplification efficiency was heterogeneous between the tested gene loci, ranging up to 3.8-fold between each locus.

Although a reaction time of 3 hours generated a feasible amount of DNA, the average fragment size seemed to be too short for efficient use as a template for further PCR reactions, even with an amplicon size ranging from 100 bp to 200 bp. In contrast with that, published data for subsequent whole-genome sequencing suggests that this relatively small amplicon size may be sufficient.^{8,10,14,22} The published range bias generated with whole-genome amplification methods lies between six-fold and eight-fold.^{8,10,14,18,22} In comparison with that, the methodical bias of IMDA, even for FFPE tissue, is, significantly below.^{5,10}

The relatively high bias in our experiment can be explained by the thickness of the tissue slides (1–4 μm); during microdissection, nucleoli may be fragmented, leading to a loss of genetic loci in individual cells. Moreover, all diagnostically relevant genes and mutations were detected after IMDA preamplification. Subsequent sequencing gave evaluable results using two independent sequencing methods (capillary sequencing and pyrosequencing).

New challenges in the upcoming years will require new molecular biological techniques that are currently not established in routine pathology laboratories. Therefore, isothermal amplification methods, originally

derived from microbiological work-up processes,^{12,22–24} are merely examples of the potential new techniques for modern pathology that can be derived from other fields of research. In this context, the outstanding role of isothermal amplification methods has to be mentioned. Additionally, it is of note that non-thermostable DNA polymerases avoid artificial variants because of their proofreading ability and simultaneously enable the advantages of non-thermostable DNA polymerases.^{4,6,11,13,15–19,21} Nevertheless, IMDA is not suitable for rapid on-site evaluation during the biopsy process because of the long reaction time. Molecular diagnostics is an evolving field in routine pathology. Therefore, the development and integration of feasible, sensitive, robust and efficient techniques like IMDA is mandatory.

Conclusion

With a reaction time of 8 hours, a feasible amount of amplicons with adequate size for qualitative PCR can be generated using IMDA, starting with input material equaling 25 human cells. Further, tissue slides can be stained with hematoxylin and eosin for microdissection, making it much easier for the pathologist/technical assistant to find tumor cells. Additionally, this method also works with macrodissected (microscope and scalpel) tissue of a surface extension less than 1 mm^3 . For larger tissue samples, routine amplification without IMDA remains the first choice, due to the relatively high costs and additional processing time of IMDA. However, it needs to be borne in mind that every additional processing step promotes more or less bias. Nevertheless, IMDA has proven to be a feasible and robust molecular pathological method to preamplify hitherto hardly analyzable clinical specimens.

Disclosure

The authors report no conflicts of interest in this work.

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