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Trypsin and Reduction Method to Prepare DNA from Formalin-Fixed and Paraffin Embedded Samples for Methylation Analysis

Yu-Chen Han,
Department of Pathology, University Of Pittsburgh School Of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261

Yan P. Yu,
Department of Pathology, University Of Pittsburgh School Of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261

George C. Tseng, and
Department of Biostatistics, University Of Pittsburgh School Of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261

Jian-Hua Luo
Department of Pathology, University Of Pittsburgh School Of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261

In recent years, emerging evidences indicate that epigenetic alteration in the promoter region or the 5' untranslated region of genes is one of the most common molecular events associated with human malignancies[1]. To analyze methylation patterns in human malignancies, large numbers of samples are needed. Most of the human issues, however, are in the archived form of formalin-fixed and paraffin embedded (FFPE)blocks. Currently, methylation specific PCR or methylation sequencing requires the usage of DNA samples extracted only from fresh or frozen tissue. DNA extracted from FFPE tissues is of poor quality largely due to containing DNA-DNA and DNA-protein cross-links that survive the DNA extraction process. These cross-links interfere with bisulfite deamination of cytosine, and produce inconsistent, and sometimes, outright false positive results. As a result, there are only sporadic reports of epigenetic analysis of FFPE samples thus far. This greatly limits the usefulness of these samples for epigenetic analysis. A new method that eliminates these cross-links is needed for analyzing the methylation patterns of these samples. In this report, we generate a new method that has significant improvement on reducing the false positive or false negative rate of sodium bisulfite deamination.

In this study, we retrieve modifiable genomic DNA by incorporating trypsin digestion, cross-linking reduction, protein striping and DNA denaturing into a four-step process (figure 1a). In the first step, the microdissected FFPE samples are treated with 0.25% trypsin at 37°C for 30 minutes to digest DNA associated proteins and to reduce the complexity of DNA-proteins complex. This process is followed by incubating the sample with lysis buffer (80 mM Tris/HCl pH 6.8, 10 % Mercaptoethanol, 200 mM NaCl, 2 % SDS) in boiling condition for 30 minutes. This step intends to denature protein, to reduce DNA-protein cross-linking, and to remove proteins from genome DNA. The samples are then treated with phenol/chloroform to remove the reduced/dissociated proteins. The DNA is subsequently purified through ammonia acetate/ethanol precipitation.

To whom correspondence should be addressed: Jian-Hua Luo, Department of Pathology, Scaife S-760, University of Pittsburgh, Pittsburgh, PA 15261. Telephone: 412-648-8791; luoj@msx.upmc.edu.

In order to validate this methodology, we fixed fresh PC-3 cells with formalin and subsequently created a paraffin block containing these PC-3 cells. Five microns thin sections of PC-3 cell block were sliced onto glass slides. The DNA from these sections was extracted using trypsin/reduction protocol. An aliquot of fresh PC-3 cells from the same batch of culture was used as controls to determine the status of gene methylation. The DNA was treated with sodium bisulfite. Methylation PCRs were performed on each DNA preparation using degenerative methylation primers specific for MT1e, MT1h and SOX4. The PCR products from each preparation were ligated into TA cloning vector, and transformed into *E.coli*. Five colonies from each preparation were analyzed for CpG island methylation status. To evaluate the accuracy of this method, we used the methylation sequencing results from fresh prepared PC-3 cell samples as the standard. When there is a complete concordance between FFPE and fresh samples, the accuracy of the method reaches 100%, and the error rate is 0%. Vice versa, when there is no concordance between FFPE cells and fresh samples, the accuracy will be 0%, and error rate will be 100%. As shown in Table 1, the overall accuracy for trypsin/reduction method is 99.3% for MT1e, 99.4% for MT1h and 100% for SOX4. Trypsin/reduction has only 4 errors in total. To investigate the utility of this method in analyzing methylation status of genes of archived tissues, we selected five samples of FFPE tissues with relatively long storage time (10 to 20 years) and their matched frozen counterpart. Subsequently, we performed methylation PCR and methylation sequencing on these samples as described above. The results from FFPE samples were then compared with those from matched frozen tissues to evaluate the accuracy of the methylation analysis. As shown in table 1, the accuracy rate of cytosine deamination ranges from 97% to 100% for the CpG islands of these 3 genes, appears not to be affected by long storage time. As a result, the trypsin/reduction method presented in the study appears reproducible and reliable for analyzing FFPE archived tissues. In order to compare our method with the existing Heat/alkaline[2] and Heat/proteinase K[3] methods, we extracted DNA from the FFPE PC3 cell blocks using these methods, and performed methylation analysis on MT1e and MT1h genes. As shown in table 2, the overall error rate of heat/alkaline is 3%, while heat/proteinase K is 9.5%, significantly higher than that of trypsin/reduction method (0.6%, $p=0.003$ versus heat/alkaline, $p=1.7 \times 10^{-12}$ versus heat/proteinase K). Furthermore, trypsin/reduction method appears to accurately analyze the methylation status of FFPE tissues with long storage time, making this method highly useful in analyzing archived clinical samples.

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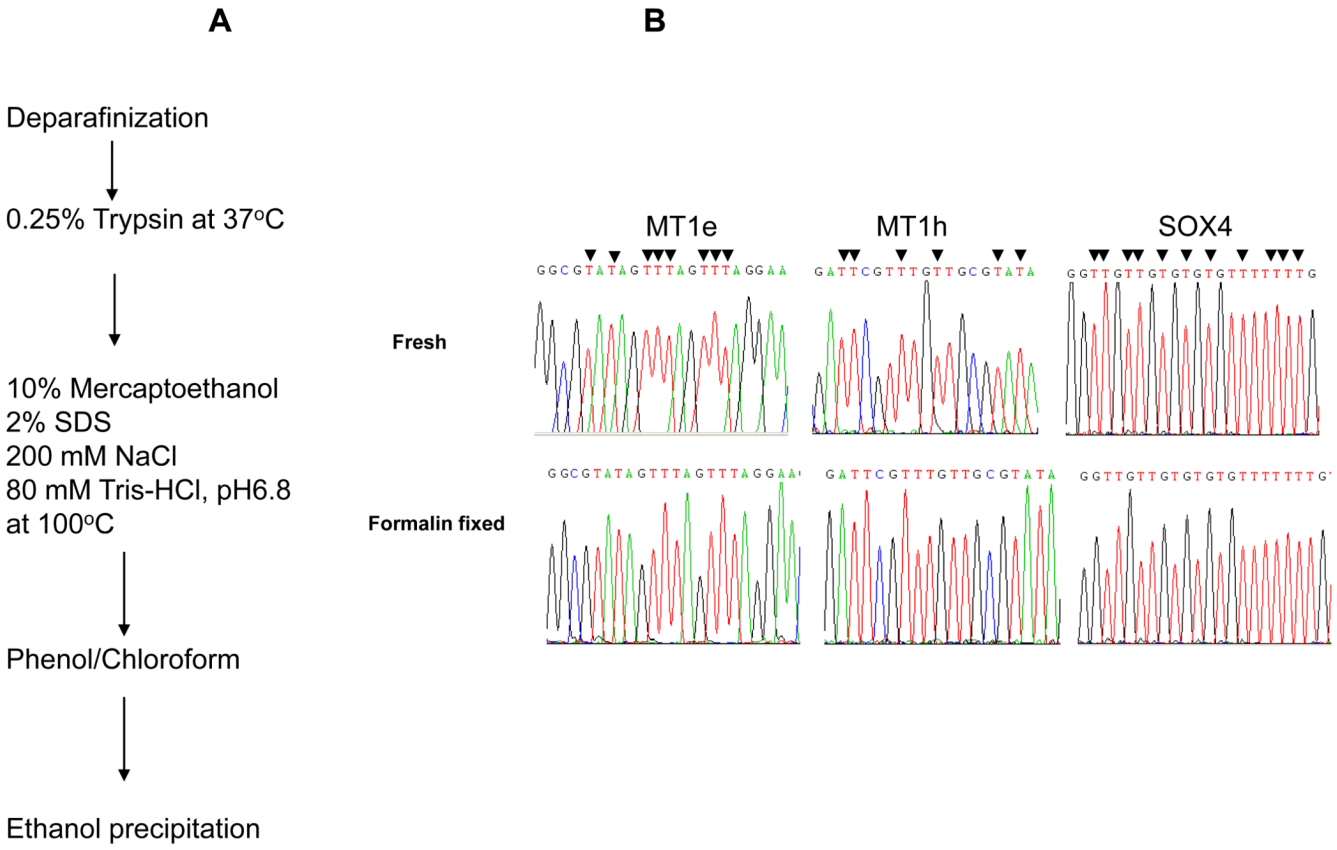

Figure 1. Trypsin and reduction protocol for formalin-fixed and paraffin-embedded samples

(A) Flow-chart of Trypsin/reduction protocol. (B) Examples of methylation sequencing results of MT1e, MT1h and SOX4 on sodium bisulfite treated DNA from fresh sample, formalin-fixed and paraffin-embedded samples. Arrows indicate conversion of cytosine to thymidine.

Table 1

Utility of trypsin and reduction method in formalin-fixed and paraffin-embedded tissues

Sample	Years in storage	MT1e	MT1h	SOX4
		% correct	% correct	% correct
FFPE PC3*	1 year	99% (278/280)	99% (348/350)	100% (195/195)
#52	10 years	100% (56/56)	100% (70/70)	97% (38/39)
#67	10 years	96% (54/56)	100% (70/70)	100% (39/39)
#73	15 years	100% (56/56)	97% (68/70)	100% (39/39)
#75	20 years	100% (56/56)	99% (69/70)	100% (39/39)
#94	20 years	100% (56/56)	100% (70/70)	100% (39/39)

*Results of five blocks.

Table 2

Detail error rate of methodology

Error	Trypsin/reducing		Heat/alkaline		Heat/proteinase K	
	MT1e	MT1h	MT1e	MT1h	MT1e	MT1h
Over-conversion	2.1% (2/95)	2% (2/100)	5.3% (5/95)	5% (5/100)	5.3% (5/95)	6% (6/100)
Under-conversion	0% (0/185)	0% (0/250)	3.8% (7/185)	0.8% (2/250)	14.1% (26/185)	9% (23/250)
Total	0.7% (2/280)	0.6% (2/350)	4.3% (12/280)	2% (7/350)	11% (31/280)	8.3% (29/350)