Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Optimization of DNA isolation method from Formalin-Fixed-Paraffin-Embedded tissues (FFPE) and comparative performance of four different Polymerase Chain Reaction (PCR) kits

Almira Zada[1,2], Puspasari S. Susanto[2], Raden L. Putri[3], Edhyana K. Sahiratmadja[1,2], Bethy S. Hernowo[4], Andri Rezano[5], Yunia Sribudiani[1,2*]

[1] Department of Biomedical Sciences, Division of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
[2] Research Center of Medical Genetics, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
[3] Genetics and Molecular Biology Laboratory, Faculty of Medicine, Universitas Padjadjaran, Bandung 40161, Indonesia
[4] Department of Pathology Anatomy, Dr. Hasan Sadikin General Hospital, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
[5] Department of Biomedical Sciences, Division of Cell Biology, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia

Correspondence to: y.sribudiani@unpad.ac.id, yungfbp@gmail.com

Received August 31, 2018; **Accepted** October 15, 2018; **Published** October 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.13.8

Abstract: The tissue sample may have important genetic information in diagnostic, prognostic and counselling issues. Formalin-Fixed-Paraffin-Embedded (FFPE) is a routine method for preserving tissues. However, DNA isolated from FFPE tissue is often difficult to be amplified in PCR due to fragmentation and DNA-protein crosslinks. This study aimed to optimize the DNA isolation method from FFPE tissue and compare the performance of four different PCR ready-to-use kits. Genomic DNA was isolated from FFPE tissue colon of Short-segment Hirschsprung (S-HSCR) patients and prostate cancer tissue using Quick-DNA™ FFPE Kit (Zymo Research) with and without pre-heating treatment in KOH/NOH solution. Primers for *Androgen Receptor (AR)* gene and four different PCR kits: MyTaq HS Red Mix 2X (BioLine), FastStart Taq DNA Polymerase (Roche), KAPA2G fast PCR Kit 2X (KAPA Biosystem) and KOD FX Neo (Toyobo) were used for amplification. DNA electrophoresis was performed to compare the PCR results. BioLine and Toyobo kits gave better PCR results than those of Roche and KAPA Biosystem. Increasing amount of Taq polymerase and dNTPs of Roche kit by two-fold could increase the quality of PCR results. Toyobo could amplify DNA up to 417 bp, however, none of these PCR kits could amplify DNA above 450 bp. Pre-heated treatment of FFPE tissue in NaOH/KOH did not improve the DNA quality and PCR results. Toyobo PCR ready-to-use kit gave the best result among the other three PCR kits used in this study in amplifying DNA isolated from FFPE tissue. Designing the primers producing amplicon not more than 450 bp is suggested.

Key words: Amplification**;** DNA-FFPE; DNA Isolation; PCR kit; Tissue.

Introduction

The tissue sample may have important genetic information in diagnostic, prognostic and counselling issues. Formalin-fixation has great importance in clinical histology and widely used to preserve tissue especially cancer tissue for routine histopathological diagnostics. Furthermore, DNA extracted from Formalin-Fixed-Paraffin-Embedded (FFPE) is crucial for retrospective clinical research (1). However, DNA FFPEs most of the time, are difficult to be amplified by Polymerase Chain Reaction (PCR) method (2,3). Many DNA extraction protocols in several types of sample from fresh or FFPE tissues, cells or whole blood have been studied. However according to our knowledge there is no commercially available PCR kit yet which specifically use DNA extracted from the FFPE tissue (4,5). Formalin-fixation leads to the formation of DNA-protein crosslinks, which increase the sensitivity of DNA to mechanical stress and decrease the accessibility to the enzymes (6-8). Formalin also could be oxidized to formic acid which, under stronger acidic conditions, purines are liberated resulting in degraded or fragmented DNA (7). Duration of fixation, type of fixative agent, the composition of fixative agent (formalin concentration, pH, and salt concentration), tissue type and temperature also contribute to DNA degradation (9-11). Those factors contribute to the poor performance of DNA-FFPE PCR. To overcome the cross-linkages that unfortunately occurs in formalin-fixed tissues, the previous study has shown a simple heating method based in alkali solution on the antigen retrieval principle commonly used in an attempt to apply Immunohistochemistry (IHC) staining to archival paraffin sections (8,12). It was reported that these similar cross-linkages might have some analogies in the retrieval of nucleic acids from protein such as in IHC (8). In this study, the nucleic acids retrieval using alkali solution (NaOH or KOH 0.05M and 0.1M) in high temperature (pre-heating) DNA isolation method was performed as in an attempt to overcome PCR inhibition caused by crosslinking and compared the results with the non-heating method. Furthermore, four different PCR commercial kits were used to compare which commercially available PCR kit work best on DNA isolated

from FFPE tissue.

Materials and Methods

Tissues

Formalin-fixed paraffin embedded (FFPE) tissue block from colon of S-HSCR and prostate cancer patients were used in this study. Seventeen FFPE tissue blocks from Prostate Cancer patients and 16 FFPE tissue blocks from S-HSCR patients were obtained from Department of Pathology Anatomy, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia, during a period from 2014 to 2017.

Design of study

This study was an experimental study using DNA isolated from FFPE tissue for sample and DNA isolated from leucocyte for positive control. Ethical approval was obtained from the Faculty of Medicine Universitas Padjadjaran Ethical Committee in using preserved biological samples.

Genomic DNA isolation from leucocytes

Genomic DNA was extracted from three-millilitre peripheral blood using Genomic DNA isolation kit (Roche Life Sciences) and the MagNA Pure LightCycler32 instrument (Roche Life Sciences) according to the manufacturer's protocol. The DNA was eluted with 50 µl of 1x TE Buffer. The DNA concentration was measured using NanoDrop™ 1000 (Thermo Scientific).

Genomic DNA isolation from Formalin-Fixed Paraffin-Embedded (FFPE) tissue

Genomic DNA from FFPE isolated using DNA™ FFPE Kit (Zymo Research) according to the manufacturer's protocol with and without pre-heated treatment prior to DNA isolation. Pre-heating treatment was performed by incubating 5-10 slices of 10nm thick sections of FFPE Tissue for 30 minutes in NaOH or KOH buffer solution with different concentration (0,05 M or 0,1 M) and heated at 80°C, 100°C or 120°C using a water bath, heat block and autoclave respectively to achieve the desired temperature. DNA concentration was measured using NanoDrop™ 1000 (Thermo Scientific).

DNA restoration treatment

Fifty microliters of pre-PCR DNA restoration treatment solution (125 µl Tween-20 (100%), 75 µl Tris HCl 1M, 75 µl MgCl2 25 mM, 25 µl dNTPs 10mM, 1000 µl Mili Q) was mixed with 10 µl of FFPE DNA isolated from colon tissue in PCR microtube. This mixture was incubated at 55° C for 1 hour, placed on the ice block and 1 unit of *Taq* DNA Polymerase was added. DNA polymerization performed at 72° C for 20 minutes and samples can be stored at -20°C for further PCR reaction. Just before PCR amplification, the denaturation step was performed by incubating 10 µl of the restored solution at 95° C for 5 minutes and then immediately chilled on ice. PCR amplification then performed as described.

Polymerase Chain Reaction (PCR) using DNA FFPE colon tissue

Touch-down PCR was conducted with the annealing temperature at 68°C to 58°C in a total volume of 25 µl containing 3 µl of DNA FFPE colon tissue for PCR template ranging from 25 ng to 290 ng in concentration, 1 µl (10 µM) of each primer to amplify exons regions of *Androgen Receptor* (*AR*) gene (Table S1) and Mili Q water with various commercial PCR Kit that has been used for comparison (Table S2): MyTaq HS Red Mix 2X (BioLine, London, UK), FastStart *Taq* DNA Polymerase, dNTPack (Roche, Basel, Switzerland), KAPA2G fast PCR Kit 2X (KAPA Biosystem, Wilmington, Massachusetts, USA), and KOD FX Neo (Toyobo, Osaka, Japan). Cycling conditions used were a Touch-Down PCR program with primer annealing temperature (Tm) started from 68°C and lowered by 1°C at every cycle for 10 cycles to 59°C then continued at 58°C for 20 cycles. (Table S3)

Results

PCR - using FFPE DNA isolated without pre-heating treatment

PCR optimization was performed using 4 different commercial kit: MyTaq HS Red Mix 2x (BioLine, London, UK), KAPA2G Fast PCR Kit (KAPA Biosystem, Wilmington, Massachusetts, USA), FastStart *Taq* DNA Polymerase, dNTPack (Roche, Basel, Switzerland) and KOD FX Neo (Toyobo, Osaka, Japan). PCR optimization result using primer to amplify exon 8 of *AR* gene (320 bp) with various commercial PCR kit as mentioned above without pre-heating treatment upon the high concentration FFPE DNA samples (≥ 100 ng/µl) revealed that BioLine and Toyobo kit held similar best result followed by Roche and KAPA kit (Figure 1A and B). FFPE DNA samples with low concentration (< 100 ng/µl) with or without DNA restoration treatment could not be amplified with either of the kits used for this study. Polymerase Chain Reaction using Roche PCR kit with doubled amount of dNTPs and Taq Polymerase from standard protocol revealed better amplification product in low concentration (<100 ng/µl) FFPE DNA (Figure 2). Polymerase Chain Reaction result has revealed as that BioLine and Toyobo PCR kit gave the best result for FFPE DNA amplification for ≥ 100 ng/µl in concentration with the PCR product size around 320 bp. Further optimization was performed using Toyobo PCR kit with a various exon of AR gene primer as followed: exon 5 (285 bp), exon 8 (320 bp), exon 7 (417 bp), exon 2 (458 bp), exon 1B (579 bp) and exon 1A (629 bp). This optimization revealed that the size of the DNA region can only be amplified up to 417 bp which was seen in exon 7 of AR gene primer used

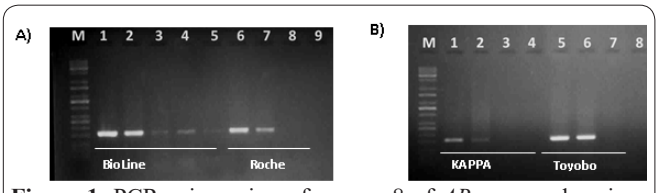

Figure 1. PCR using primer for exon 8 of *AR* gene and various concentration of FFPE DNA. **A)** Bioline (2: ≥ 100 ng/µl; 3: < 100 ng/µl; 4: pre-PCR restoration DNA treatment) and Roche (7: ≥ 100 ng/µl; 8: < 100 ng/µl) **B)** KAPA Biosystem (2: ≥ 100 ng/µl; 3: < 100 ng/µl) and Toyobo (6: ≥ 100 ng/µl; 7: < 100 ng/µl). Leucocyte DNA was used for positive control (well 1 and 6 in gel A and well 1 and 5 in gel B). The negative control was shown in well 5 and 9 in gel A and well 4 and 8 in gel B. M: DNA ladder/marker.

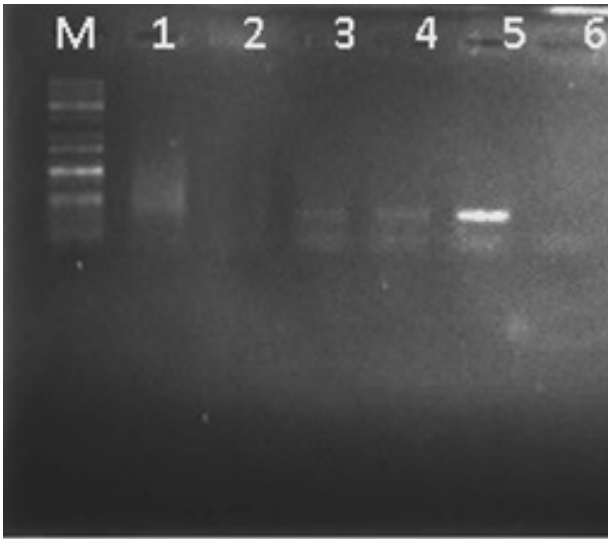

Figure 2. PCR using primer for exon 8 of AR gene with a low concentration of FFPE DNA (< 100 ng/µl) using Roche PCR kit. (1), DNA concentration 100 ng/µl with standard protocol of dNTPs and Taq Polymerase amount (2), DNA concentration < 100 ng/µl with standard protocol of dNTPs and Taq Polymerase amount (3), DNA concentration < 100 ng/µl with doubled amount of Taq Polymerase (4), DNA concentration < 100 ng/µl with doubled amount of dNTPs and (5), DNA concentration < 100 ng/µl with doubled amount of dNTPs and Taq Polymerase. M: DNA ladder/marker. (6) Negative control.

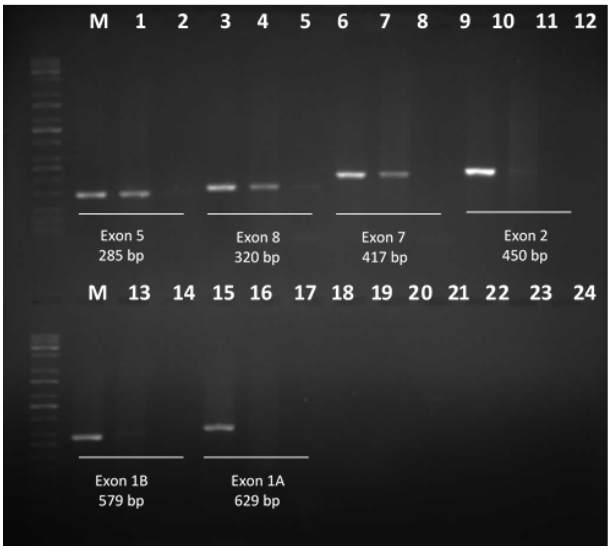

Figure 3. PCR performed using DNA from leucocyte, FFPE DNA high concentration (≥ 100 ng/µl) and low concentration respectively for exon 5 (well: 2-3), exon 8 (well:5-6), exon 7 (well:8-9), exon 2 (well:11-12), exon 1B (well:14-15) and exon 1A (well:17-18). Positive control using DNA leukocyte in well 1,4,7,10,13,16. Negative control of each respective exon as mentioned above (well:19-24). M: DNA ladder/marker. Toyobo kit gave the best results for DNA isolated from FFPE as it can be amplified DNA region up to 417 bp in size well: 8).

(Figure 3). Similar experiments were performed using the other three kits, but the maximum PCR products could be amplified were only 320 bp (Figures were not shown).

PCR Optimization - Pre-Heating FFPE DNA isolation treatment

PCR optimization was performed using MyTaq HS Red Mix 2x (BioLine, London, UK) commercial kit

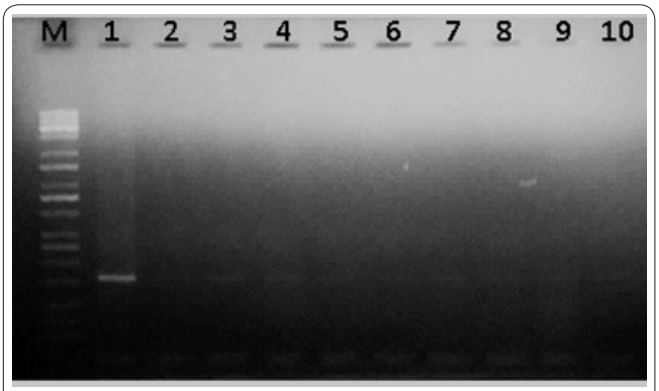

Figure 4. PCR performed using FFPE DNA with pre-heating DNA isolation treatment in the various buffer. Well 2-5 PCR using pre-heating DNA isolation treatment with NaOH 0,05 M buffer at 80OC (2 and 3), 100 OC, and 120 OC. Well, 6-8 have preheated DNA isolation treatment with KOH 0,05 M buffer at 80 OC, 100 OC, and 120OC respectively. Well, 9 is pre-heated DNA isolation treatment with deparaffinization solution (Zymo Research) at 80OC. Well 1: DNA isolated from leucocyte as a positive control. Well 10: negative control. M: DNA ladder/marker.

with exon 8 of AR gene primer (320 bp) was used for amplification. The DNA used for this amplification was an FFPE DNA which undergoes pre-heating DNA isolation treatment with NaOH or KOH 0,05M buffer based on DNA concentration measurement that had shown better DNA concentration results than the other buffer used in this study. The amplification result revealed that none of the pre-heating treatment of FFPE DNA with different buffer gave satisfying results on PCR optimization (Figure 4).

Discussion

DNA isolation method used in this study was a modification from that described in the previous study by Shi SR, et al, 2004. Combination of high temperature and alkaline pH solution in extracting DNA from FFPE tissue may denature and hydrolyze proteins leads to rupture of the cell membrane and nuclear membrane, as well as breakage of DNA-protein crosslink due to formalin fixation (8,12). However, this method had a different outcome in this study. Possible reasons could be that the preparation of FFPE tissue was different in term of the concentration of formaldehyde buffer used, the duration of fixation time and also the age of FFPE tissue block used in this study. The older the FFPE tissue block might introduce more damage to DNA as the oxidation of paraffin resulted in formic acid which leads to DNA fragmentation. Also, in this study, commercial DNA extraction kit was used instead of the conventional protocol using Phenol-Chloroform Isoamyl-Alcohol (PCI) DNA purification that had been used in the previous study. Previous studies showed that DNA from FFPE tissues is severely fragmented. This condition leads to insufficient "good quality DNA" for DNA amplification using PCR especially for large amplicons (13,14). The unstable amplification also due to an inhibitory effect of DNA FFPE itself. Fragmentation process generated short DNA debris that might act directly as DNA polymerase inhibitor (1). In certain condition, increasing the amount of nucleotides concentration (dNTPs) or the *Taq Polymerase* enzyme concentration could overcome

the PCR inhibition. The doubled concentration of both dNTPs and polymerase (using Roche kit) proved to increase PCR robustness in this study.

Furthermore, to get a better PCR results we tried to restore the low concentration and the quality DNA FFPE using the restoration treatment as mentioned in the method section in order to modify the damaged site allowing subsequent copying of template. However, this step did not improve the quality of DNA and resulted in good PCR product as we expected. The pH and the composition of the restoration buffer might not provide an optimum condition for *Taq polymerase* to amplified DNA and filling the gap on the damage sites. Further study needs to be done to get the optimum pH and buffer composition to restore the damage on DNA FFPE.

Toyobo kit (15) and Bioline kit (16) gave the satisfactory result on amplifying high concentration DNA FFPE compared to the Kapa Biosystem kit (17) dan Roche kit(18). Toyobo (KOD FX Neo) kit contains DNA polymerase isolated from the hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1. This kit also contains elongation enhancers which allow greater efficiency of elongation capability up to 40 kb human genomic DNA. Bioline (My Taq HS Red Mix) enabling amplification of very low amount DNA due to its MyTaq HS DNA Polymerase and a novel buffer system. The author suggested that the DNA polymerase and the enhancers contained in both kits were sufficient to amplify high concentration DNA FFPE. Among four PCR kits used in this study, Toyobo performed the best as it could amplify DNA FFPE up to 417 bp in size.

The authors conclude that doubled the concentration of PCR reagents (*Taq* polymerase and dNTPs) in general could enhance the quality of PCR product. In this study, it has been shown that Toyobo gave the best PCR results on DNA isolated from FFPE tissue compared to the other three kits, as it can amplify DNA region up to 417 bp. In addition, designing primers which produce amplicon not more than 450 bp in size is suggested.

Acknowledgements

The authors thank the Head of Laboratory Dr. Ani M. Maskoen and staffs of Genetics and Molecular Biology Laboratory, Faculty of Medicine, Universitas Padjadjaran for their assistance during the experiments. The authors thank Rony Lesmana, MD., PhD for allowing us to use the heating block for this experiment.

Conflict of interest disclosure

The authors stated there is no conflict of interest regarding the researches, authorship, and/or publication of this article. None of the authors was affiliated to the companies mentioned in this article.

Funding statement

For experiment from Porstate Cancer Tissue was funded by Universitas Padjadjaran Internal Grant 2017 (Riset Fundamental UNPAD) and for experiment from colon tissue of Hirschsprung disease patient was funded by Hibah Penelitian Terapan Unggulan Perguruan Tinggi (PTUPT) Kemenristek Dikti 2017.

References

1.Dietrich D, Uhl B, Sailer V, Holmes EE, Jung M, Meller S et al. Improved PCR Performance Using Template DNA from Formalin-Fixed and Paraffin-Embedded Tissues by Overcoming PCR Inhibition. PLoS ONE 2013; 8(10):e77771.

2. Bereczki L, Kis G, Bagdi E, Krenacs L. Optimization of PCR amplification for B- and T-cell clonality analysis on formalin-fixed and paraffin-embedded samples. Pathol Oncol Res 2007;13:209-14.

3. Webster JD, Miller MA, DuSold D, Ramos-Vara J. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. J Histochem Cytochem 2009; 57(8):753-61.

4. Fallah F, Minaei Chenar H, Amiri H, Omodipur S, Shirbande Ghods F, Kahrizi D, et al. Comparison of two DNA extraction protocols from leave samples of *Cotinus coggyria, Citrus synensis* and *Genus juglans*. Cell Mol Biol 2017;63(2):76-8.

5. Ghaheri M, Kahrizi D, Yari K, Babaei A, Suthar RS, Kazemi E. A comparative evaluation of four DNA extraction protocols from whole blood sample. Cell Mol Biol 2016; 61(3):119-23.

6. Kuykendall JR, Bogdanffy MS. Efficiency of DNA-histone cross-linking induced by saturated and unsaturated aldehydes in vitro. Mutat Res 1992;283(2):131-6.

7. Bonin S, Petrera F, Niccolini B, Stanta G. PCR analysis in archival postmortem tissues. Mol Pathol 2003;156:184-6.

8. Shi SR, Datar R, Liu C, Wu L, Zhang Z, Cote RJ et al. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: Heat-induced retrieval in alkaline solution. Histochem Cell Biol 2004;122(3):211-8.

9. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161(6):1961-71.

10. Zsikla V, Baumann M, Cathomas G. Effect of buffered formalin on amplification of DNA from paraffin wax embedded small biopsies using real-time PCR. J Clin Pathol 2004,57,654-6.

11. Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD et al. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when?. PLoS ONE 2007;2(6): e537.

12. Shi SR, Cote RJ, Wu L, et al. DNA Extraction from Archival Formalin-fixed, Paraffin-embedded Tissue Sections Based on the Antigen Retrieval Principle: Heating Under the Influence of pH. J Histochem Cytochem 2002;50(8):1005-11

13. Bielawski K, Zaczek A, Lisowska U, et al (2001). The suitability of DNA extracted from formalin-fixed, paraffin-embedded tissues for double differential polymerase chain reaction analysis. Int J Mol Med 2001;8(5):573-8.

14. Inadome Y, Noguchi M. Selection of higher molecular weight genomic DNA for molecular diagnosis from formalin-fixed material. Diagn Mol Pathol 2003;12(4):231-6.

15. Toyobo. KOD FX Neo [Internet]. Toyobo Co., LTD. 2017 (cited 2 October 2018). Available from: http://www.toyobo-global.com/seihin/xr/lifescience/support/manual/KFX-201.pdf

16. Bioline. My Taq™ HS Red Mix [Internet]. 2017 (cited 2 October 2018). Available from: https://www.bioline.com/mytaq-hs-red-mix.html

17. Kapa Biosystem. Kapa 2G Fast HotStart Ready Mix PCR Kit [Internet].2017 (cited 2 October 2018). Available from: https://www.kapabiosystems.com/document/kapa2g-fast-hotstart-readymix-tds/?dl=1

18. Roche. FastStart Taq DNA Polymerase, dNTP Pack [Internet]. 2017 (cited 2 October 2018). Available from: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Roche/Bulletin/1/fpcrnrobul.pdf