Application Note

High-quality genomic DNA isolation and sensitive mutation analysis

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Introduction

A major objective of cancer research is to gain insight into genetic disorders and alterations, including somatic mutations and rearrangements. The goal can be finding new drug targets or expanding our understanding of underlying mechanisms of drug resistance. In vitro analyses of the responses of cancer cell lines to drugs reveal a considerable amount about the effects of mutations. Due to the heterogeneity of patient-derived cell lines in terms of mutation frequency, it is expected that treatment responses will differ.

For the results of such studies to have real meaning, it must be possible to detect somatic mutations at the lowest possible level consistently. Thus, it is essential to have efficient and reliable genomic DNA isolation and PCR technology, especially if cells from heterogeneous tumors are to be cultured and used in dose–response and ADMET experiments.

This study assessed the yield and overall quality of genomic DNA isolated from four cancer cell lines and admixtures thereof with a wild-type cell line. The aim was to ensure that the chosen method, which used the QIAamp® DNA Mini Kit for gDNA isolation and the qBiomarker® Somatic Mutation PCR Arrays and Assays for detection of mutation status, would reliably and sensitively detect even very low percentage mutations.

Materials and Methods

Cell culture

The cell lines were cultured in 75 cm^2 tissue culture flasks at 37°C and 5% CO_2 until a cell density of 80% confluence was reached. The cell culture media are listed consecutively in Table 1.

Table 1. The cell culture media for each cancer cell line used in the study

Cell line	Background	Medium
A431	Human epidermoid carcinoma cells	500 ml DMEM complete[1] + 50 ml FCS[2] + 5 ml MEM[3] + 5 ml L-glutamin[4] + 5 ml penicillin and streptomycin[5]
HT29	Human colorectal adenocarcinoma cells	500 ml DMEM complete[1] + 50 ml FCS[2] + 5 ml MEM[3] + 5 ml L-glutamin[4] + 5 ml penicillin and streptomycin[5]
H1975	Human non-small cell lung cancer cells	500 ml RPMI[6] + 50 ml FCS[2] + 5 ml L-glutamin[4] + 5 ml penicillin and streptomycin[5]
MCF7	Human breast cancer cells	500 ml RPMI[6] + 50 ml FCS[2] + 5 ml MEM[3] + 5 ml L-glutamin[4] + 5 ml penicillin and streptomycin[5] + 5 ml Pyruvat[7] + 1.25 ml insulin[8]

[1] Gibco 31885-023
[2] Biochrom S-0415
[3] Gibco 11140-035
[4] Gibco 25030-024
[5] Sigma P-0781
[6] Gibco 31870-025
[7] Gibco 11360-039
[8] Gibco 12585-014

To harvest the cells and to adjust the cell admixtures, the media was removed and the cells were washed with PBS. After a short treatment (10–30 sec) with 1 ml trypsin (Sigma T-3924), the cells were removed from the plate using a cell-scraper, then pelleted by centrifugation (2 min, 300 x *g*, RT) and washed with PBS. The cells were centrifuged again and adjusted to 1×10^6 cells/ml in PBS. The cell admixtures were prepared by mixing the appropriate volume of the cancer and wild-type cell lines. The admixtures were centrifuged again and the supernatant was removed. Genomic DNA was immediately isolated from each pellet.

Genomic DNA isolation

Genomic DNA was isolated from aliquots of 1×10^6 cells using a QIAamp DNA Mini Kit and the protocol for cultured cells. The gDNA was eluted in a volume of 80 µl. The gDNA yields, concentration and A_{260}/A_{280} ratio were determined using a NanoDrop® Spectrometer based on the A_{260} and A_{280} readings. The gDNA integrity was analyzed on 1% agarose gel.

Mutation analysis

Each sample was tested for the presence of mutations using the Human Lung Cancer qBiomarker Somatic Mutation PCR Array or mutation-specific qBiomarker Somatic Mutation PCR Assays. The reaction mix for a 4 x 96-well array was prepared by adding 200 ng of input DNA to 550 µl qBiomarker Probe Mastermix and nuclease-free water to a final volume of 1100 µl. Samples corresponding to 2 ng gDNA (10 µl reaction mix) were transferred to each well of the array. The tested samples were run in duplicate.

Table 2 shows the reaction setup used to detect a single somatic mutation. The tested samples were run in triplicate using the single assays. Table 3 shows the cycling conditions. Cycling was done on a Thermo Fisher Scientific® ViiA™ 7 Real-Time PCR System with 384-Well Block. FAM fluorescence was recorded from every well during the annealing and extension step of each cycle. The base line was set at cycles 8–20 and the thresholds were set manually at 0.04 for the array and 0.01–0.04 for the single assays.

Table 2. The reaction setup for mutation detection

Component	Volume
qBiomarker Probe Mastermix	10 µl
qBiomarker Somatic Mutation PCR Assay	1 µl
DNA sample	30 ng
Water	Variable
Total volume per sample	10 µl

Table 3. Cycling conditions for the reaction

Step	Time	Temperature	Number of cycles
Initial PCR activation step	10 min	95°C	1
2-step cycling:			1 µl
Denaturation	15 sec	95°C	40
Annealing and extension	60 sec*	60°C	

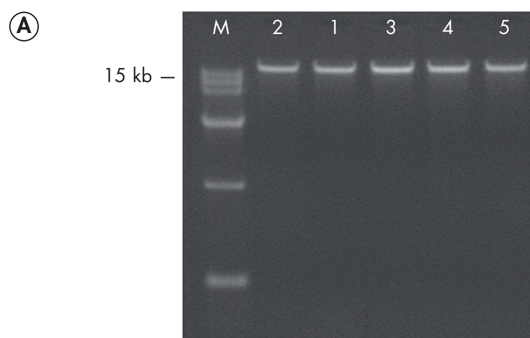
Data analysis

The data analysis of the arrays was performed using free data analysis software for qBiomarker Somatic Mutation PCR Arrays (**www.SABiosciences.com/somaticmutationdataanalysis.php**). The basis was the $\Delta\Delta C_T$ method, which is described in detail in (1). Basically, the ΔC_T for each mutation assay in each sample (Test) is calculated as the difference between the mutation assay ($C_T^{GeneMUT}$) and the corresponding gene copy reference assay ($C_T^{GeneREF}$; e.g., BRAF V600E assay C_T and BRAF copy assay C_T). The same assays are run with a wild-type sample (WT) with the wild-type alleles of the appropriate gene and the ΔC_T for WT is calculated accordingly. When ΔC_T^{TEST} is significantly smaller than ΔC_T^{WT} ($\Delta C_T^{TEST} < \Delta C_T^{WT}$) according to statistical analysis or a preset threshold (e.g., 4 cycles), a positive mutation call ("+") can be made.

Results

The mutation call accuracy for each cancer cell line was checked. Genomic DNA was isolated from 1 x 10^6 cells from each cancer cell line using the QIAamp DNA Mini Kit. The gDNA yield was found to be consistent, in the range of 7–12 µg for all the cell lines, and sufficient for the PCR array analysis. The A_{260}/A_{280} ratios were in the range of 1.8–1.9 indicating a high purity of the extracted DNA. The agarose gel analysis showed high molecular weight gDNA for all the tested cell lines indicating intact, non-degraded gDNA. The gDNA integrity and quality results are shown in Figure 1.

A



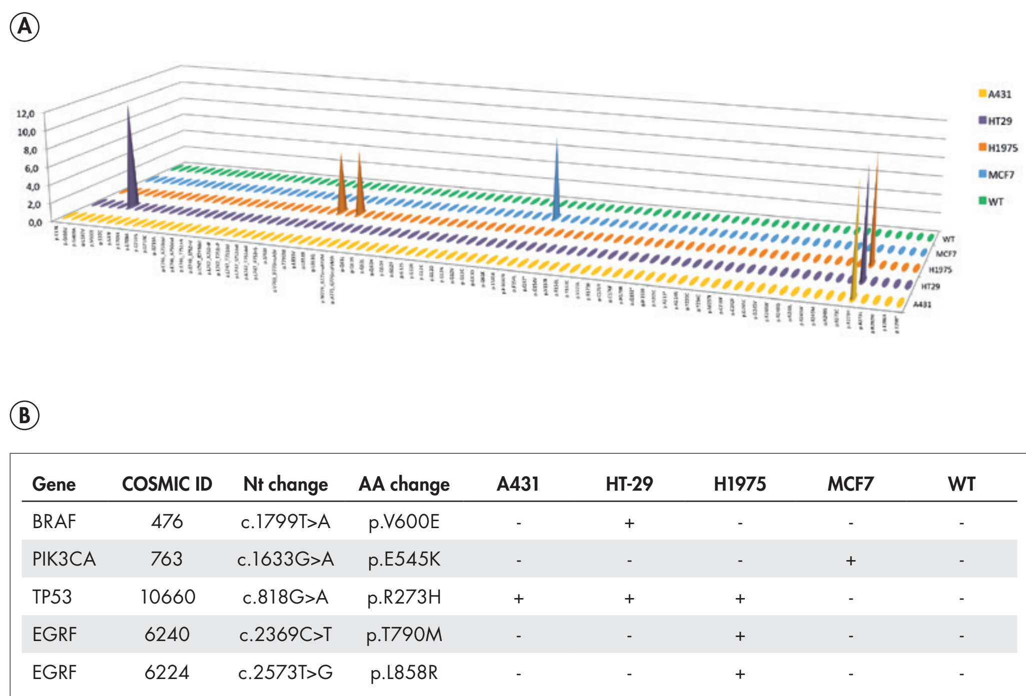
B

Cellline	Mean concentration (ng/µl)	Mean yield (µg)	Mean $_{260}/_{280}$ ratio
A431	89.6 ± 25.5	7.2 ± 2.0	1.8 ± 0.1
HT29	147.2 ± 14.5	11.8 ± 1.2	1.9 ± 0.1
H1975	131.2 ± 15.4	10.5 ± 1.2	1.8 ± 0.0
MCF7	149.1 ± 21.8	11.9 ± 1.7	1.8 ± 0.0
WT	99.8 ± 15.3	8.0 ± 1.2	1.8 ± 0.0

Figure 1. The high quality and yield of genomic DNA isolated from cancer cell lines using the QIAamp DNA Mini Kit. A Agarose gel analysis showing that high molecular-weight genomic DNA was obtained from all the cell lines Lane 1 – A431, lane 2 – HT-29, lane 3 – H1975, lane 4 – MCF7, lane 5 – wild-type. **B** Mean yields of genomic DNA isolated from the cell lines. The consistent 260/280 ratio indicates consistent quality.

We checked the cancer cell lines for the putative mutations using the Human Lung Cancer qBiomarker Somatic Mutation PCR Array, which allows the screening of 85 mutations intimately connected to lung cancer in human specimens. Importantly, it is not restricted for this type of cancer. Some of the genes represented of the array are also present in other cancer cell lines and therefore of interest in this study. The used wild-type reference cell line carries the wild-type alleles for the somatic mutations that can be analyzed using this array.

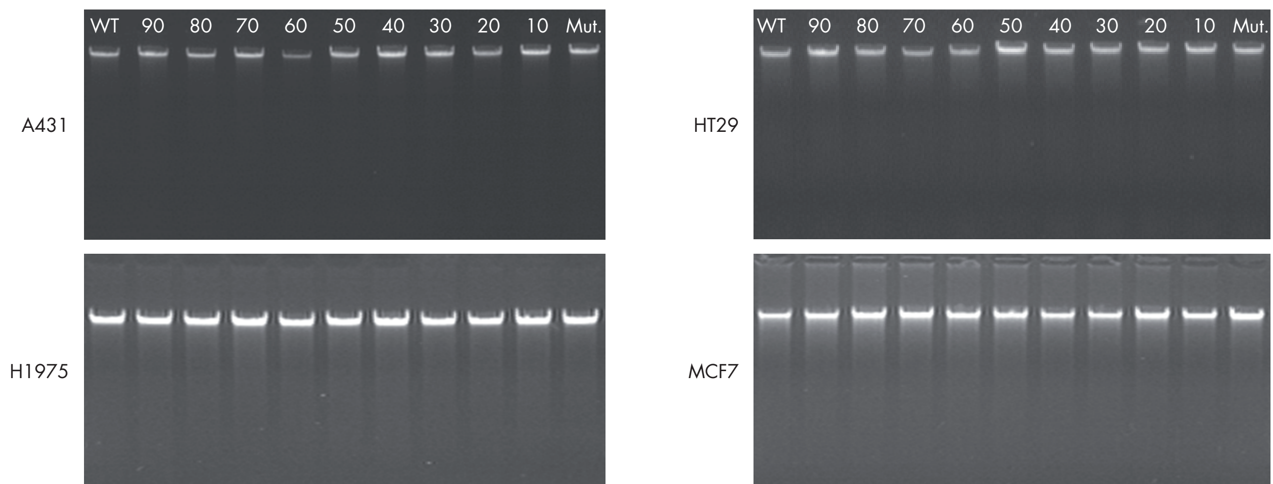
The C_T values were below the C_T cutoff for all mutations, meaning that the Human Lung Cancer qBiomarker Somatic Mutation PCR Array detected the five supposed mutations in the cancer cell lines. No mutation was detected in the wild-type cell line. Figure 2A shows the result of the array analysis and the detection of five common somatic mutations within the four cancer cell lines. Based on the $\Delta\Delta C_T$ method, a call ≥4 could be calculated for the corresponding assays, indicating the presence of the somatic mutation within the cancer cell line (Figure 2B). The amplification curves for each mutation were comparable to the curves for the copy number and positive control, meaning high-quality input DNA and no inhibitors (data available on request). All of the assays resulted in a C_T value clearly below the C_T cutoff of 35 and no relevant signal was detected with the wild-type DNA. The mutation assay for TP53 in the wild-type cell line generated a C_T of 39, which is clearly distant from the cutoff.



Gene	COSMIC ID	Nt change	AA change	A431	HT-29	H1975	MCF7	WT
BRAF	476	c.1799T>A	p.V600E	-	+	-	-	-
PIK3CA	763	c.1633G>A	p.E545K	-	-	-	+	-
TP53	10660	c.818G>A	p.R273H	+	+	+	-	-
EGRF	6240	c.2369C>T	p.T790M	-	-	+	-	-
EGRF	6224	c.2573T>G	p.L858R	-	-	+	-	-

Figure 2. Detection of somatic mutations using qBiomarker Somatic Mutation PCR Array in cancer cell lines. A The five common somatic mutations were detected in the four cell lines. No mutation was detected in the wild-type line (WT). **B** All of the described mutations for each cell line were called correctly (+ : positive call ≥ 4).

In order to detect the mutation in a background of wild-type gDNA, we prepared a series of cell line admixtures from wild-type (WT) and cancer cell lines. Cells of the wild-type cell line were diluted with cells of the single cancer cell line to give WT to cancer cell line ratios from 100% WT to 100% cancer cell line in steps of 10%. The gDNA were extracted from all cell line admixtures in duplicate. The results for the gDNA quality control and the results for the real-time PCR are presented in Figure 3.



B

Cell line	Conc. (ng/µl)		260/280		Yield (µg)	
	Mean	SD	Mean	SD	Mean	SD
A431	47.7	8.9	1.9	0.1	3.8	0.7
HT29	80.2	18.9	1.9	0.0	6.4	1.5
H1975	144.6	19.1	1.9	0.0	11.6	1.5
MCF7	47.0	6.3	1.9	0.2	3.8	0.5

Figure 3. Uniformity of gDNA quality and mutation detection from different cancer cell line admixtures. A Agarose gel analysis indicating reproducible DNA integrity for the different cell line admixtures WT: 100% wild-type cells. Mut: 100% cancer cell line. Ratio of WT to cancer cell line indicated by number above other lanes. **B** Results of spectroscopic measurement revealing comparable DNA yield and quality range for the cell line admixtures. DNA extraction was performed for each 100% cell line and all cell line admixtures in duplicate. The range of the obtained values is reported and the mean from all samples from each individual cell line was calculated. **C** The assays on the array were able to detect all mutations in all cell line admixtures at 10% except the BRAF V600E, EGFR T790M and L858R mutations, which were detected at 20%.

C

Cell line	Gene	Nt change	WT	10% mut.	20% mut.	30% mut.	40% mut.	50% mut.	60% mut.	70% mut.	80% mut.	90% mut.	100% mut.
A431	TP53	c.818G>A	-	+	+	+	+	+	+	+	+	+	+
HT-29	BRAF	c.1799T>A	-	-	+	+	+	+	+	+	+	+	+
	TP53	c.818G>A	-	+	+	+	+	+	+	+	+	+	+
H1975	TP53	c.818G>A	-	+	+	+	+	+	+	+	+	+	+
	EGRF	c.2369C>T	-	-	+	+	+	+	+	+	+	+	+
	EGRF	c.2573T>G	-	-	+	+	+	+	+	+	+	+	+
MCF7	PIK3CA	c.1633G>A	-	+	+	+	+	+	+	+	+	+	+

To test sensitivity and to validate mutation call accuracy, the 100% cancer cell, the 100% wild-type cell lines and 6 admixtures mimicking 6 mutant samples containing 50, 20, 10, 5, 2.5 and 1% of the tested cancer cell lines were analyzed using single mutation-specific qBiomarker Somatic Mutation PCR Assays, which have higher sensitivity as they allow higher input template amounts (from 4–30 ng). The assays detected all mutations in all admixture samples and the mutation call could be verified down to the 1% level (Figure 4A). As an example, the corresponding amplification curves of the analyzed 1% cell dilution for the BRAF V600E and TP53 R273H mutation in HT29 as well as the PIK3CA E545K mutation in MCF7 are shown in Figure 4B. There is a clear C_T value difference at the 1% level between the mutation assay and the C_T cutoff of 35.

Conclusions

The number of cells with important mutations can be very small in heterogenic cancer cell samples, so the complete recovery of genomic DNA is essential if maximum molecular insight is to be achieved. The studied workflow, based on genomic DNA isolation using the QIAamp DNA Mini Kit and sensitive mutation detection using the qBiomarker Somatic Mutation PCR Assays, proved highly effective. The isolated DNA was free of inhibitors and robust and sensitive detection of even low percentage mutations.

References

1. Long, S., You, M., Wang, L. and Wang, Y. (2012) Rapid and accurate cancer somatic mutation profiling with the qBiomarker Somatic Mutation PCR Arrays. Published by QIAGEN and available at **http://www.sabiosciences.com/manuals/qBiomarkerSomaticMutation.pdf**

A

Mut. Cell	Gene	Nt change	100% mut.	50% mut.	20% mut.	10% mut.	5% mut.	2,5% mut.	1% mut.	100% WT
A431	TP53	c.818G>A	+	+	+	+	+	+	+	-
HT-29	BRAF	c.1799T>A	+	+	+	+	+	+	+	-
	TP53	c.818G>A	+	+	+	+	+	+	+	-
H1975	TP53	c.818G>A	+	+	+	+	+	+	+	-
	EGRF	c.2369C>T	+	+	+	+	+	+	+	-
	EGRF	c.2573T>G	+	+	+	+	+	+	+	-
MCF7	PIK3CA	c.1633G>A	+	+	+	+	+	+	+	-

B

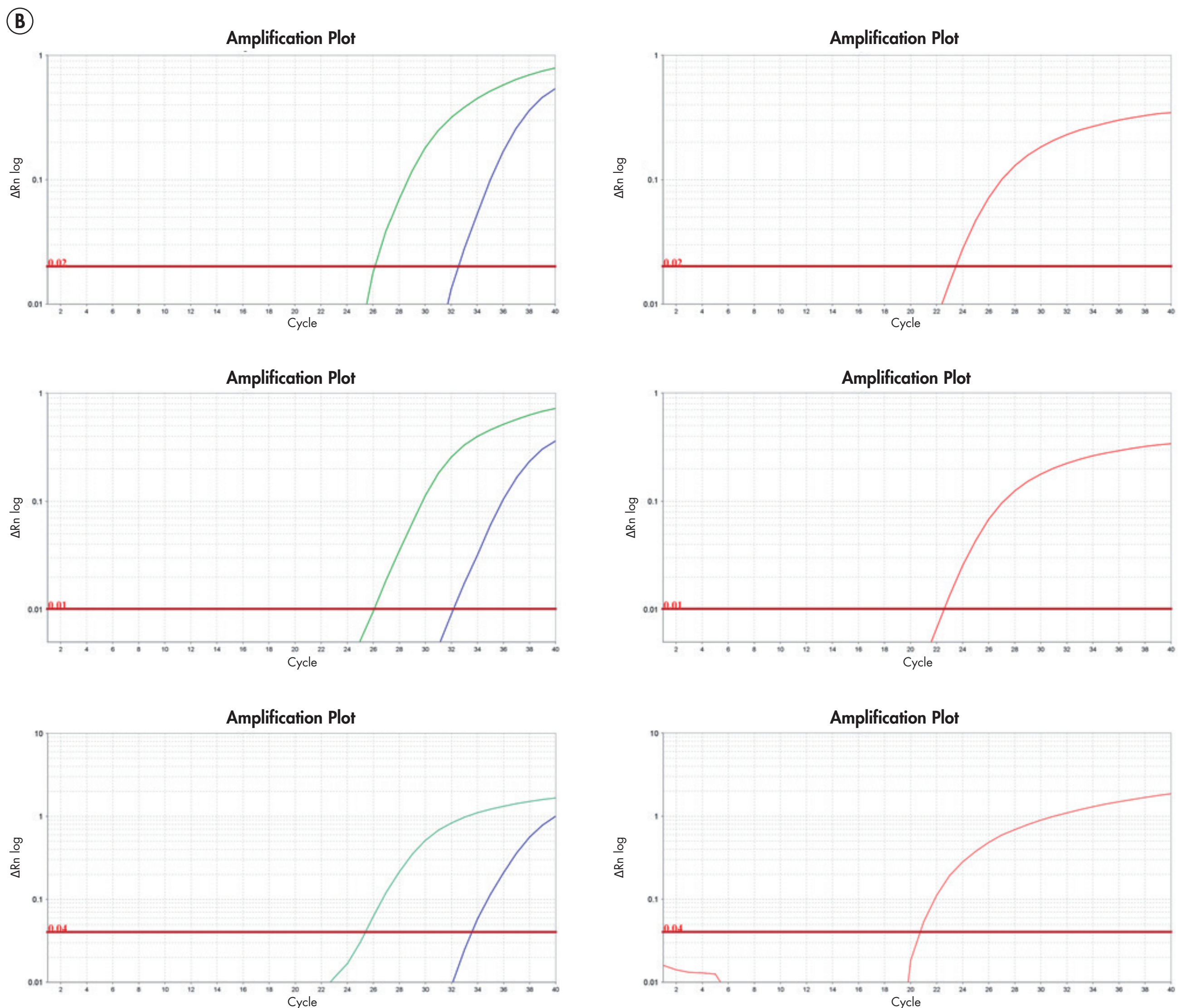


Figure 4. Detection of somatic mutations using qBiomarker Somatic Mutation PCR Assays. To test sensitivity the genomic DNA was extracted from cell line admixtures mimicking different mutant samples containing 100%, 50%, 20%, 10%, 5%, 2.5% and 1% each of the above cancer cell line in a wild-type cell line background. The mutations were detected using single qBiomarker Somatic Mutation Primer Assays for the single mutations. **A** All tested mutations could be detected in the different cell line admixtures at 1%. **B** Amplification plots for the BRAF V600E and TP53 R273H mutation in HT29 as well as the PIK3CA E545K mutation in MCF7 (blue curves) and the corresponding copy number controls (green curves) and positive controls (red curves) showing typical curves enabling accurate C_T value determination and sensitive detection of low levels of mutant DNA.

Ordering Information

Product	Contents	Cat. no.
qBiomarker Somatic Mutation PCR Arrays	PCR plate and Mastermix	337021
qBiomarker Somatic Mutation PCR Assays	PCR assay and Mastermix	337011
QIAamp DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304

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