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digene® HPV Genotyping RH Test, Detection Kit Handbook

Σ 20

Version 1

IVD

For detection of 18 high-risk human papillomavirus (HPV) genotypes by reverse hybridization

CE

REF 613413

HB

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Contents

Kit Contents

***digene* HPV Genotyping RH Test, Detection Kit**			**(20)**
Catalog no.			**613413**
Number of reactions			**20**
	High-Risk HPV Strips		20
DS	Denaturation Solution	CONT NaOH	250 µl
HS	Hybridization Solution		85 ml
SW	Stringent Wash Solution		2 x 105 ml
C	100x Conjugate	CONT ProClin 300	550 µl
CD	Conjugate Diluent		55 ml
S	100x Substrate		550 µl
SB	Substrate Buffer		110 ml
RS	5x Rinse Solution		75 ml
3B	3B Buffer		220 µl
	Incubation Trough Frames		3
	Data Reporting Sheets		2
	Handbook	HB	1

Symbols

Contains sufficient for <N> tests

In vitro diagnostic medical device


European conformity

Catalog number


Manufacturer

Batch code

Material number


Important note


Temperature limitations


Use by


Consult instructions for use

Storage

All reagents in the *digene* HPV Genotyping RH Test, Detection Kit should be stored immediately upon receipt at 2–8°C. Make sure to store the kit away from any source of contaminating DNA, especially amplified DNA products. All reagents are stable until their expiration date under these conditions. High-Risk HPV Strips are stable until their expiration date when stored desiccated at 2–8°C.

Intended Use

The *digene* HPV Genotyping RH Test, Detection Kit is an in vitro reverse-hybridization assay using GP5+/6+ amplimers for the qualitative identification of individual high-risk HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. The use of this test is indicated as a reflex test for women with a positive result obtained with the *digene* HC2 High-Risk HPV DNA Test.

Product Use Limitations

The test has been validated for use with specimens collected in Specimen Transport Media (STM) and PreservCyt® Solution. Testing of other specimen types may result in false negative or false positive results. The product should be used by personnel trained in PCR techniques. The recommendations on laboratory design and procedures must be followed to avoid false results and DNA contamination. The hybridization and stringent wash incubations in the manual test procedure should be performed at exactly 50°C to prevent false positive or false negative results. The temperature of the waterbath must be verified with a calibrated thermometer.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *digene* HPV Genotyping RH Test, Detection Kit is tested against predetermined specifications to ensure consistent product quality.

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the *digene* HPV Genotyping RH Test, Detection Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

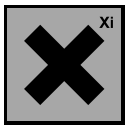
For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx, where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

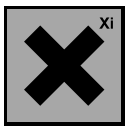
The following risk and safety phrases apply to components of the *digene* HPV Genotyping RH Test, Detection Kit.

Denaturation Solution (DS)



Contains sodium hydroxide: irritant. Risk and safety phrases:* R36/38, S26-36/37/39-45.

Conjugate, 100x (C)



Contains ProClin® 300 (5-chloro-2-methyl-4-isothiazolin-3-one, 2-methyl-4-isothiazolin-3-one): irritant. Risk and safety phrases:* R43, S24-36/37/39-45.

* R36/38: Irritating to eyes and skin; R43: May cause sensitization by skin contact; S24: Avoid contact with the skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection; S45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The *digene* HPV Genotyping RH Test is comprised of 2 kits: the *digene* HPV Genotyping RH Test, Amplification Kit and the *digene* HPV Genotyping RH Test, Detection Kit. The *digene* HPV Genotyping RH Test, Detection Kit enables easy and reliable identification of high-risk human papillomavirus (HPV) genotypes by reverse hybridization. PCR products used in the reverse hybridization procedure are generated from template DNA by amplification of the highly conserved L1 sequence using the *digene* HPV Genotyping RH Test, Amplification Kit.

Principle

Identification of HPV genotypes is based on a reverse hybridization procedure. Denatured biotinylated amplicons, resulting from amplification of part of the L1 region with the GP5+/6+ primer set, are hybridized with specific oligonucleotide probes, which are immobilized as parallel lines on membrane strips (Figure 1). After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added, which binds to any biotinylated hybrid present. Incubation with BCIP/NBT chromogen yields a purple precipitate which permits visual interpretation of results. As an internal control for the presence of amplifiable DNA after isolation, a fragment from the human beta-globin gene is co-amplified with the HPV DNA in the form of a multiplex PCR. The last probe line on the strip contains a probe capable of detecting the beta-globin amplimer.

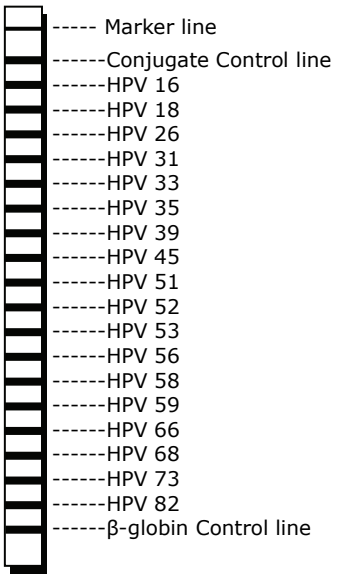


Figure 1. Schematic of the genotyping strip. A marker line (**Marker line**) is drawn at the top of the strip for orientation. Two control lines (**Conjugate Control line**, **β-globin Control line**) are included.

Figure 2. Workflow of the *digene* HPV Genotyping RH Test, Detection Kit, manual procedure.

Hybridization in shaking waterbath at 50°C, closed lid

Denaturation Solution (DS)	10 µl	add into each test trough
3B Buffer (3B)	10 µl	add into drop of Denaturation Solution (DS)
Amplified product	10 µl	add into drop of Denaturation Solution (DS)

Incubation at room temperature; 5 minutes

Hybridization Solution (HS)*	2 ml	add to denatured sample, mix gently
HPV Strip	1 strip	add into each test trough
*(prewarmed)		

Incubation at 50°C; 60 minutes

⬇

Stringent wash in shaking waterbath at 50°C, closed lid

Stringent Wash (SW)*	2x2 ml	wash strips 2 times for 10 to 20 seconds
Stringent Wash (SW)*	2 ml	add into each test trough
*(prewarmed)		

Incubation at 50°C; 30 minutes

ⓘ Prepare 1x rinse solution and conjugate solution

⬇

Color development on shaker, at room temperature

1x Rinse solution	2x2 ml	rinse strips 2x1 minute
(conc. Rinse Solution (RS), 1/5 in water)		
Conjugate Solution	2 ml	add into each test trough
(conc. conjugate, 1/100 in Conjugate Diluent [CD])		

Incubation at room temperature; 30 minutes

ⓘ Prepare substrate solution

⬇

(continued on next page)

Figure 2. Workflow of the *digene* HPV Genotyping RH Test, Detection Kit, manual procedure (continued from previous page).

Color development on shaker, at room temperature		
1x Rinse solution (conc. Rinse Solution (RS), 1/5 in water)	2x2 ml	rinse strips 2x1 minute
Substrate Buffer (SB)	2 ml	rinse strips 1x1 minute
Substrate Solution (conc. substrate, 1/100 in Substrate Buffer [SB])	2 ml	add into each test trough
Incubation at room temperature; 30 minutes		
Distilled water	2x2 ml	add to the troughs of the incubation tray
Incubation at room temperature; 3 minutes		
Interpretation and storage of the strips		
ⓘ This is a procedure summary of the test which can be used as a guide. It should not be used as a substitute for the detailed protocol as described in this handbook.		

Performance characteristics

Analytical sensitivity

Formal Limit of Detection (LOD) determinations have been carried out for HPV 16, HPV 18, and HPV 45. The detection limit is shown in Table 1.

Table 1. Analytical sensitivity limit of detection (LOD) for HPV 16, 18, and 45

Genotype	LOD concentration (copies/PCR)
HPV16	4
HPV18	8
HPV45	23

For the remaining 15 types, the dilution steps have been analyzed and the concentration with 100% positive result was determined. The results are shown in Table 2.

Table 2. Sensitivity limit of detection (LOD) concentration as a function of genotype

Genotype	LOD concentration (copies/PCR)
HPV26	1000
HPV31	10
HPV33	10
HPV35	10

Table continued on next page.

Table 2. Continued

Genotype	LOD concentration (copies/PCR)
HPV39	1000
HPV51	1000
HPV52	1000
HPV53	100000
HPV56	10
HPV58	100
HPV59	100
HPV66	100
HPV68a	10000
HPV68	100000
HPV73	10000
HPV82MM4	100000
HPV82IS39	10000

Practical analytical specificity

Amplimers from the 18 HPV genotypes and 2 subtypes targeted in the assay (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 (68a), 73, 82MM4 (82IS39)) showed a correct reaction pattern on the strip, thereby confirming the identification of the correct HPV type. High amounts of amplimer (obtained from 10,000,000 HPV copies per PCR from HPV (sub) types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 56, 58, 59, 61, 66, 67, 68 (and 68a), 69, 71, 72, 73, 81, 82 (MM4 and IS39)) were used to

trace any cross-reactivity. None of the probes showed any reaction with an amplimer from a non-targeted HPV type.

The specificity of the *digene* HPV Genotyping RH Test, Detection Kit is ensured by the selection of the probes, as well as the selection of stringent reaction conditions. The probes were checked for possible homologies to all in gene banks published sequences by sequence comparison analysis. The detectability of all relevant strains has thus been ensured. In addition microorganisms commonly found in the female anogenital tract have been analyzed. None of the tested pathogens was reactive (see Table 3). The presence of these microorganisms is not reducing the sensitivity to detect the high risk HPV genotypes.

Table 3. Potentially cross-reactive pathogens

Pathogen
Acinetobacter anitratus
Acinetobacter lwoffi
Bacteroides fragilis
Escherichia coli (HB101)
Escherichia coli
Klebsiella pneumoniae
Lactobacillus acidophilus
Mobiluncus curtisii
Mobiluncus mulieris

Precision

Initially, a panel of ten different samples, containing plasmids of all 20 HPV genotypes and subtypes covered by the assay, was tested at ten times above the estimated detection limit in triplicate on 3 different days by 2 technicians and

once by 2 other kit lot numbers, resulting in a total of 24 data points per sample. All genotyping results were identical.

Then, DNA purified from a total of 92 samples was tested by two technicians using three lots of kits. Results were scored as either identical (100% matching genotypes), compatible (at least one genotype in common) or discordant (no matching genotypes). Between the two technicians, the percentage of identical/compatible agreement was between 98% and 100%. When comparing the different lots of kits that were used by both technicians (i.e., comparing all 6 outcomes), the percentage of identical/compatible agreement was 93%.

Finally, a selection of 25 samples from the panel above was tested in duplicate by two technicians (inter-technician testing) at a second testing site (inter-laboratory testing). Results were again scored as identical (100% matching genotypes), compatible (at least one genotype in common) or discordant (no matching genotypes). Technician 1 showed 100% identical agreement in the duplicate testing of the 25 samples. The second technician achieved 93% identical agreement (23/25 samples) in the duplicate samples, with 2 samples showing compatible genotyping results.

Between the two technicians, the percentage of identical agreement was 88%, and the percentage of compatible agreement was 12%. No discordant results were observed. When comparing all ten genotyping results from each of the 25 samples in the inter-laboratory testing panel (first and second site), the percentage of identical/compatible agreement was 100% and comprised 72% identical results and 28% compatible results. No discordant results were seen.

In summary, to assess the closeness of agreement between measurements, a series of artificial samples containing all HPV types covered by the assay and 92 clinical samples were tested by multiple sampling. Each sample was assayed in replicates on different days by different technicians. Also different lots were tested. The results showed 100% of identical genotypes in artificial samples and more than 95% identical and compatible results for clinical samples.

Accuracy

Aliquots of DNA purified from a total of 108 samples comprising 50 HC2-positive samples collected in STM, 50 HC2-positive samples collected in PC and 8 HC2-negative samples in STM were tested with the *digene* HPV Genotyping RH Test, Detection Kit and the Free University RLB system (1).

Results were scored as either identical (100% matching genotypes), compatible (at least one genotype in common), or discordant (no matching genotypes). Discrepancies (discordant genotyping results) were resolved by repeating both assays and, in case of remaining discrepancies, by subsequent analysis with a third sensitive HPV detection and genotyping assay [SPF10-LiPA$_{25}$ (version1)].

The comparison yielded 80% identical, 11% compatible genotyping results, and 9% of discordant genotyping results. Repeating both assays resolved 5 of the 10 discrepant samples. Subsequent analysis with the SPF10-LiPA$_{25}$ (version 1) resolved another 3 discrepant samples leaving only two samples discrepant. In the first of these discrepant samples, HPV45 was found by the *digene* HPV Genotyping RH Test, Detection Kit whereas the reverse line blot (RLB) was negative. In the second sample, HPV58 was detected with the RLB and the *digene* HPV Genotyping RH Test, Detection Kit result was negative.

In summary, to assess the closeness of agreement the *digene* HPV Genotyping RH Test, Detection Kit was tested against the HPV RLB assay, an accepted reference value. Parallel testing of 108 clinical samples were done and the results were scored as identical, compatible, or discordant. Discordant samples were resolved by analysis with the SPF10-LiPA$_{25}$ (version 1). The results showed a very low level of discrepant samples (2%) after resolution of initial discrepant samples. See Table 4.

Table 4. Testing of HPV genotyping test against HPV RLB assay

Accuracy analysis	% of clinical samples
Identical	80
Compatible	18
Discrepant	2

Robustness

The capacity of the assay to remain unaffected by deliberate variations in relevant method parameters was assessed as follows. Duplicate samples of HPV genotypes 16, 18, 45, and 52 at 10 times the detection limit of the PCR were amplified with PCR conditions 1.0°C higher or 1.0°C lower. HPV detection by the RH test was not influenced as compared to normal cycling conditions.

Amplimers with a concentration close to the detection limit of the RH detection kit were tested at higher hybridization temperatures to investigate possible loss of signals. Testing at maximum specified temperature of 50.5°C did not influence the result and testing at 51.0°C (which exceeds test specifications) resulted in slightly fainter reactive probe lines but no loss of signal. High concentrations of amplimers were tested at lower hybridization temperatures to investigate possible cross-reactions. Testing was at both 49.5°C (which is the indicated lower temperature limit for hybridization), and at 49.0°C (which is below the specification); no cross-reactions were observed. Reducing all incubation times of the RHA procedure by five minutes resulted in slightly fainter reactive probe lines but no loss of signal, whereas elongating all incubation times by five minutes did not have any visible effect.

Interfering substances

The effect of blood and other potentially interfering substances was evaluated in the *digene* HPV Genotyping RH Test, Detection Kit. The presence of the agents (named in Table 5) did not cause false-positive results.

In addition, the presence of the substances did not cause a reduced sensitivity of the HPV viruses.

Table 5. Interfering substances

Substance
Blood
Moisturizer
Hemorrhoidal anesthetic
Talcum Powder
Anti-fungal cream
Vaginal lubricant

Diagnostic evaluation

The *digene* HPV Genotyping RH Test was compared with the established Free University RLB system (1) in a multicenter study.

For this purpose, 267 *digene* HC2 High Risk HPV DNA Test positive cervical scrapes were selected. The samples were collected in STM and PreservCyt medium. The DNA from these samples was isolated either manually with the QIAamp® MinElute® Virus Spin Kit or automated on the EZ1 Advanced with the EZ1® DSP Virus Kit.

Of the 267 samples, 254 of the HC2 positives samples were also positive for one of the 18 HR HPV types by digene HPV Genotyping RH Test.

Assay common genotypes were compared and results were scored as either identical (100% matching genotypes), compatible (at least one genotype in common), or discordant (no matching genotypes).

The percentage of identical/compatible agreement between the genotyping assays was 95.5 % (255 of 267 samples) with 82.0 % identical and 13.5 % compatible results (see Table 6). 4.5 % (12 of 267 samples) of the genotyping results were discordant.

Table 6. Comparison of genotyping findings between the *digene* HPV Genotyping RH Test and the established Free University RLB System in 267 HC2 positive cervical smears.

Agreement	N=267 (%)
Identical typing result	219 (82.0)
Compatible typing result	36 (13.5)
Discordant typing result	12 (4.5)

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For all protocols

- Distilled water or deionized water
- Pipets* and disposable sterile pipet tips with filters (1–20 µl, 20–200 µl, and 200–1000 µl)
- Forceps for handling strips
- Graduated cylinders (10 ml, 25 ml, 50 ml, and 100 ml)

Manual protocol

- Waterbath* with inclined lid and shaking platform (80 rpm); capable of attaining 50 ±0.5°C
- Aspiration device
- Calibrated thermometer*
- Orbital shaker or rocking platform
- Laboratory timer, 2 hours (±1 minute)
- Optional: Multidispenser (for example, Finnpipette® Stepper from Thermo Electron, see www.thermo.com)*†

Automated protocol

- ProfiBlot™ 48 T (Tecan Trading, AG; see www.tecan.com)*†
- 50 ml conical tubes

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

† This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Important Notes

Preparing reagents for the manual procedure

1x Rinse solution

Prepare a 1/5 dilution of 5x Rinse Solution (RS) by adding one part 5x Rinse Solution (RS) to 4 parts distilled or deionized water. Prepare 8 ml diluted Rinse Solution (1x) for each test trough plus an extra 10 ml (see Table 7).

Table 7. Preparation of 1x rinse solution (for manual process)

No. of strips	Volume of 5x Rinse Solution (RS) (ml)	Volume of water (ml)	Total volume (ml)
1	3.6	14.4	18
5	10	40	50
10	18	72	90
15	26	104	130
20	34	136	170

Substrate Buffer (SB)

Use 2 ml Substrate Buffer (SB) for each strip, plus an extra 5 ml for each run. To reduce the excess Substrate Buffer (SB) required for each run, use a 50 ml conical tube and place it in the bottle provided with the ProfiBlot 48 T.

Conjugate solution and substrate solution

To obtain working solutions of conjugate and substrate, dilute 100x Conjugate (C) or 100x Substrate (S) 1/100 in Conjugate Diluent (CD) or Substrate Buffer (SB), respectively. Prepare 2 ml conjugate solution or substrate solution for each test trough plus an extra 2 ml (see Tables 8 and 9). Only use clean vessels that have been rinsed with distilled water for the preparation of conjugate and substrate solutions.

Table 8. Preparation of conjugate solution (for manual process)

No. of strips	Volume of Conjugate Diluent (CD) (ml)	Volume of 100x Conjugate (C) (µl)
1	4	40
5	12	120
10	22	220
15	32	320
20	42	420

Table 9. Preparation of substrate solution (for manual process)

No. of strips	Volume of Substrate Buffer (SB) (ml)	Volume of 100x Substrate (S) (µl)
1	4	40
5	12	120
10	22	220
15	32	320
20	42	420

Preparing reagents for the automated procedure

1x Rinse Solution

Prepare a 1/5 dilution of 5x Rinse Solution (RS) by adding one part 5x Rinse Solution (RS) to 4 parts distilled or deionized water. Prepare 10 ml diluted rinse solution (1x) for each strip plus an extra 55 ml (see Table 10, below).

Table 10. Preparation of 1x rinse solution (for automated process)

No. of strips	Volume of 5x Rinse Solution (RS) (ml)	Volume of water (ml)	Total volume (ml)
1	13	52	65
5	21	84	105
10	31	124	155
15	41	164	205
20	51	204	255

Substrate Buffer (SB)

Use 2 ml Substrate Buffer (SB) for each strip, plus an extra 5 ml for each run. To reduce the excess Substrate Buffer (SB) required for each run, use a 50 ml conical tube and place it in the bottle provided with the ProfiBlot 48 T.

Conjugate solution and substrate solution

To obtain working solutions of conjugate and substrate, dilute 100x Conjugate (C) or 100x Substrate (S) 1/100 in Conjugate Diluent (CD) or Substrate Buffer (SB), respectively. Prepare 2 ml conjugate solution or substrate solution for each strip plus an extra 5 ml (see Tables 11 and 12, next page). To reduce the excess required buffer required for each run, use a 50 ml conical tube and place it in the bottle provided with the ProfiBlot 48 T. Only use clean vessels for the preparation of conjugate and substrate solutions that have been rinsed with distilled water.

Table 11. Preparation of conjugate solution (for automated process)

No. of strips	Volume of Conjugate Diluent (CD) (ml)	Volume of 100x Conjugate (C) (µl)
1	7	70
5	15	150
10	25	250
15	35	350
20	45	450

Table 12. Preparation of substrate solution (for automated process)

No. of strips	Volume of Substrate Buffer (SB) (ml)	Volume of 100x Substrate (S) (µl)
1	7	70
5	15	150
10	25	250
15	35	350
20	45	450

Protocol 1: Detection of Amplified HPV DNA Using a Manual Procedure — Hybridization

Important points before starting

- Do not use reagents that have passed their expiration date.
- Do not mix reagents from different lot numbers.
- The vial containing Denaturation Solution (DS) should be closed immediately after use; prolonged exposure to air leads to rapid deterioration.
- Place all reagents and the plastic tubes containing test strips at 2–8°C immediately after use.
- Throughout the different incubation steps, the test strips should remain in the same trough.

Things to do before starting

- Allow all reagents and the plastic tubes containing the test strips to equilibrate to room temperature (15–25°C).
- Warm Hybridization Solution (HS) and Stringent Wash (SW) to at least 37°C in a waterbath. Make sure that the temperature does not exceed the hybridization temperature of 50°C. All crystals should be dissolved before use.
- Set a waterbath with inclined lid and shaking platform to 50°C. Make sure that the temperature of the water is 50 ± 0.5°C. Check the temperature using a calibrated thermometer.
- During the incubations, set the speed of the shaking waterbath as high as possible, while avoiding spillage of liquids.
- Adjust the water level in the waterbath so that it is between one-third and one-half of the height of the test trough.

Procedure

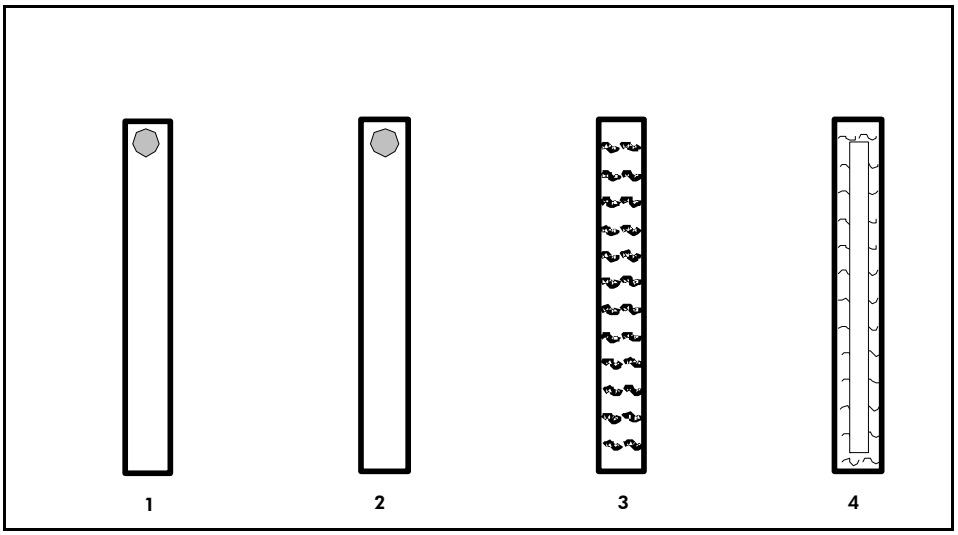


Figure 3. Pipetting instructions. 1: To trough add 10µl Denaturation Solution (DS) +10µl 3B Buffer (3B); **2**: Mix by pipetting, add 10µl amplified DNA product; **3**: Add Hybridization Solution (HS); **4**: Place strip in trough.

1. **Dispense 10 µl of Denaturation Solution (DS) into the upper corner of the required number of test troughs (1 trough per strip) (see Figure 3, 1).**
2. **Close the vial of Denaturation Solution (DS) immediately after use.**
3. **Add 10 µl of 3B Buffer (3B) to the Denaturation Solution (DS) (see Figure 3, 1).**
4. **Add 10 µl of amplified biotinylated product to the trough containing Denaturation Solution (DS) and 3B Buffer (3B).**
5. **Carefully mix by pipetting up and down a few times (see Figure 3, 2).** ⓘTake care not to contaminate neighboring troughs during pipetting. Use fresh pipet tips with filters for each sample.
6. **Incubate at room temperature (15–25°C) for 5 min.**
7. **Using forceps, remove the required number of test strips from the tube (1 strip per sample). Add an identification number above the marker line on the strip using a pencil.**

   ⓘ Do not use any other kind of writing device. Only handle the strips above the marker line using forceps. Make sure to wear gloves. Always handle the strips on paper.
8. **Shake the prewarmed Hybridization Solution (HS).**
9. **Gently add 2 ml of Hybridization Solution to the denatured amplified product in each trough (see Figure 3, 3).**

   ⓘMix by gently shaking. Take care not to contaminate neighboring troughs during pipetting.

10. **Immediately place the strip into the trough (see Figure 3, 4).**

ⓘThe strip should be completely submerged in the solution.

ⓘWear disposable, powder-free gloves and use forceps.

11. **Place the trough frame in the shaking waterbath, close the lid of the waterbath, and incubate at 50°C for 60 min.**

ⓘ Do not cover the trough frame. Condensation may result in cross-contamination between troughs.

ⓘ Do not use a hot-air shaker for the incubation. Heat transfer is not optimal and may result in aberrant results.

ⓘ Halfway through the incubation, make sure that the temperature of the water is 50°± 0.5°C. Check the temperature using a calibrated thermometer.

12. **Proceed to Protocol 2: Detection of Amplified HPV DNA Using a Manual Procedure — Stringent Wash.**

Protocol 2: Detection of Amplified HPV DNA Using a Manual Procedure — Stringent Wash

Important points before starting

- If you have completed "Protocol 1: Detection of Amplified HPV DNA Using a Manual Procedure — Hybridization," do this protocol next.
- If you are using this protocol, then you will have to do "Protocol 3: Detection of Amplified HPV DNA Using a Manual Procedure — Color Development," next.

Procedure

1. **After hybridization, remove the trough frame from the waterbath.**
2. **Hold the trough frame at an angle and aspirate the liquid from the trough using a pipet, preferably attached to a vacuum aspirator.**

   ⓘ Take care not to damage the surface of the strip below the marker line when aspirating the solution.
3. **Add 2 ml of prewarmed Stringent Wash (SW) to each trough, and rinse by shaking the trough frame at room temperature (15–25°C) for 20–30 s. Ensure that the whole strip is thoroughly washed by complete submersion in the solution.**
4. **Aspirate the solution from each trough.**
5. **Repeat the wash step once by following the instructions in steps 3 and 4 of this section. Do not allow the strips to dry between the two wash steps. Proceed to step 6 after steps 3 and 4 are repeated.**
6. **Aspirate the solution.**
7. **Add 2 ml of prewarmed Stringent Wash (SW) to each strip.**
8. **Incubate at 50°C in the shaking waterbath for 30 min.**

   ⓘ Make sure the lid of the waterbath remains closed during the incubation.

   ⓘ Do not cover the trough frame. Condensation may result in cross-contamination between troughs.

   ⓘ Do not use a hot-air shaker for the incubation. Heat transfer is not optimal and may result in aberrant results.
9. **During the incubation in step 4 of "Protocol 2: Detection of Amplified HPV DNA Using a Manual Procedure — Hybridization," prepare the 1x rinse solution and conjugate solution according to the instructions in "Preparing reagents for the manual process," starting on page 20.**
10. **At the end of the incubation, aspirate the Stringent Wash (SW).**

11. **Proceed to "Protocol 3: Detection of Amplified HPV DNA Using a Manual Procedure — Color Development."**

Protocol 3: Detection of Amplified HPV DNA Using a Manual Procedure — Color Development

Important points before starting

- If you have completed "Protocol 2: Detection of Amplified HPV DNA Using a Manual Procedure — Stringent Wash," do this protocol next.
- In this protocol, all incubation steps are performed at room temperature (15–25°C).

Procedure

1. **Add 2 ml of 1x rinse solution to each trough and incubate on a rocking platform for 1 min.**
2. **Aspirate the solution.**
3. **Add 2 ml of 1x rinse solution to each trough and incubate on a rocking platform for 1 min.**
4. **Aspirate the solution.**
5. **Add 2 of ml conjugate solution to each trough and incubate on a rocking platform for 30 min.**
6. **During the incubation, prepare substrate solution (see page 20)**

   ⓘ Protect the substrate solution from light.
7. **At the end of the incubation, aspirate the solution.**
8. **Add 2 ml of 1x rinse solution to each trough and incubate on a rocking platform for 1 min.**
9. **Aspirate the solution. Repeat step 8, once again for a total of two washes with 2 ml of 1x rinse solution.**
10. **Add 2 ml of Substrate Buffer (SB) to each trough and incubate on a rocking platform for 1 min.**
11. **Aspirate the solution.**
12. **Add 2 ml of substrate solution to each trough, cover the trough with aluminum foil, and incubate on a rocking platform for 30 min.**
13. **At the end of the incubation, aspirate the solution.**
14. **To stop color development, add 2 ml of distilled water to each trough and incubate on a rocking platform for at least 3 min.**
15. **Aspirate the solution.**
16. **Add 2 ml of distilled water to each trough and incubate on a rocking platform for at least 3 min.**
17. **Using forceps remove the strips from the troughs and place the strips on absorbent paper.**

18. **Allow the strips to dry completely. Align the conjugate control line to the corresponding line on the interpretation sheet, and adhere the strip to the sheet.**

    ⓘ Allow the strips to dry completely before visual interpretation.

19. **Store developed, dry strips at room temperature in the dark.**
20. **It is not necessary to do Protocol 4: Detection of Amplified HPV DNA Using an Automated Procedure. Proceed directly to the Troubleshooting Guide of this handbook.**

Protocol 4: Detection of Amplified HPV DNA Using an Automated Procedure

Important points before starting

- If performing the automated protocol, it is not necessary to go through the manual protocols listed in Protocols 1 — 3.
- Do not use reagents that have passed their expiration date.
- Do not mix reagents from different lot numbers.

Things to do before starting

- Allow all reagents to equilibrate to room temperature (15–25°C) before use.
- Warm Hybridization Solution (HS) to at least 37°C in a waterbath. Make sure that the temperature does not exceed the hybridization temperature of 50°C. All crystals should be dissolved before use.
  Use 2 ml of Hybridization Solution (HS) plus an extra 15 ml for each run.
- Warm Stringent Wash (SW) to at least 37°C in a waterbath. Make sure that the temperature does not exceed the hybridization temperature of 50°C. All crystals should be dissolved before use.
  Use 6 ml of Stringent Wash (SW) plus an extra 30 ml for each run.
- Prepare 10 ml of 1x rinse solution for each strip plus an extra 55 ml (for more information, see Table 9, page 21).
- Prepare 2 ml of conjugate solution and of substrate solution for each strip plus an extra 5 ml (for more information, see Tables 10 and 11, pages 22 and 23, respectively). Only use clean vessels for the preparation of conjugate and substrate solutions that have been rinsed with distilled water.
- Please use the following (Table 13, next page) to link the tubing of the appropriate channels of the ProfiBlot to the buffers.

Table 13. Placement of buffers in ProfiBlot 48T

Channel	Solution
1	Hybridization Solution
2	Stringent Wash Solution
3	Rinse Solution
4	Conjugate Diluent
5	Substrate Buffer
6	Substrate Buffer

Procedure

1. **Program the calibrated ProfiBlot 48 T using the parameters outlined in Table 14, on the next two pages.**

Table 14. The ProfiBlot 48 T program

	Parameters				
1	INC	5 min	Shake Speed: 3		
2	TEMP	**49°C**[§]			
3	DISP	Channel 1	2000 µl	Start Pos. 1	No. of Strips End
4	INC	60 min	Shake Speed: 3		
5	WASH	Channel 2	2000 µl	Start Pos. 1	No. of Strips End
6	INC	3 min	Shake Speed: 3		
7	WASH	Channel 2	2000 µl	Start Pos. 1	No. of Strips End
8	INC	3 min	Shake Speed: 3		
9	WASH	Channel 2	2000 µl	Start Pos. 1	No. of Strips End
10	INC	30 min	Shake Speed: 3		
11	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
12	COOL				
13	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
14	WASH	Channel 4	2000 µl	Start Pos. 1	No. of Strips End
15	INC	30 min	Shake Speed: 3		
16	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
17	INC	3 min	Shake Speed: 3		
18	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
19	INC	3 min	Shake Speed: 3		

Table continued on next page.

[§] The incubation temperature for hybridization is set to 49°C. In the manual procedure hybridization is performed at 50°C to obtain similar hybridization results.

Table 14. Continued

			Parameters		
20	WASH	Channel 5	2000 µl	Start Pos. 1	No. of Strips End
21	INC	3 min	Shake Speed: 3		
22	WASH	Channel 6	2000 µl	Start Pos. 1	No. of Strips End
23	INC	30 min	Shake Speed: 3		23
24	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
25	INC	3 min	Shake Speed: 3		
26	WASH	Channel 3	2000 µl	Start Pos. 1	No. of Strips End
27	INC	10 min	Shake Speed: 3		
28	ASP				
29	END				

2. **Switch on the ProfiBlot 48 T and start the program.**
   The machine will warm up until the required temperature is reached.
3. **Prepare the reagents for the assay run; see "Preparing reagents for the automated procedure," page 22, for more information.**
4. **Place the reagents into the ProfiBlot 48 T and insert the instrument tubing into the buffers. Make sure to insert the tubing into the correct buffers (see Table 13, page 32).**
5. **Using forceps, remove the required number of test strips from the tube (1 strip per sample). Add an identification number above the marker line on the strip using a pencil.**
   ⓘ Do not use any other kind of writing device. Only handle the strips above the marker line using forceps. Make sure to wear gloves.
6. **Place a marked strip into the upper corner of each trough.**
7. **Pipet 10 µl of Denaturation Solution (DS) into the lower corner of each trough that contains a strip.**
   ⓘ Close the vial immediately after use. A multidispenser can be used for distribution of Denaturation Solution (DS).

8. **Add 10 µl of 3B Buffer (3B) to the Denaturation Solution (DS).**
9. **Add 10 µl of amplified biotinylated product to the Denaturation Solution (DS) in the trough. Carefully mix by pipetting up and down a few times.**

   ⓘ Take care not to contaminate neighboring troughs during pipetting. Use a fresh, filter-plugged sterile pipet tip for each sample.
10. **Carefully place the tray in the ProfiBlot 48 T when the instrument has warmed up and start the run.**
11. **When the run has finished, wash the strips in the tray with distilled water for 5 min.**
12. **Using forceps, remove the strips from the troughs and place them on absorbent paper.**
13. **Allow the strips to dry completely.**
14. **Align the conjugate control line to the corresponding line on the interpretation sheet, and adhere the strip to the sheet.**

    ⓘ Allow the strips to dry completely before visual interpretation.
15. **Store developed, dry strips at room temperature in the dark.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Weak or no signals on all strips, including the conjugate control line	
a) **Manual procedure**: Incorrect amount of conjugate or substrate used	ⓘ Repeat the color development protocol using the same strips. Prepare freshly diluted 1x rinse solution, conjugate solution, and substrate solution.
b) **Automated procedure**: Incorrect amount of conjugate or substrate used	ⓘ Repeat the test with new strips and buffers using the same amplified material.
False-negative or excessively weak signals with the probe lines, except for the conjugate control line	
a) Inappropriate amount of amplified material added for hybridization	ⓘ The concentration of amplified material is too low due to inefficient amplification. Check the amount of amplified product by running a 10 µl aliquot on a 2% agarose gel. The length of the amplified HPV product varies between 139 and 148 bp, depending on the HPV type. See "Inefficient amplification," page 37, for more information. The beta-globin amplimer has a size of 258 bp, but will probably not be visible on gel.
b) Hybridization solution (HS) or Stringent Wash (SW) not properly heated or mixed	ⓘ Before use, warm Hybridization Solution (HS) and Stringent Wash (SW) to at least 37°C in a waterbath. Make sure that the temperature does not exceed 50°C. All crystals should be dissolved before use.

	Comments and suggestions
c) **Manual procedure**: Temperature exceeded 50°C during hybridization and stringent wash	ⓘ Make sure to set the waterbath to the correct temperature.
Inefficient amplification	
a) Too much DNA used	ⓘ Check the PCR by agarose gel electrophoresis. If too much DNA was added, a smear of DNA is often visible on the gel. Due to the low annealing temperature of the PCR some background bands on the gel are normal. Dilute the DNA and repeat the amplification by adding 10 times less DNA.
b) Insufficient amount of DNA used	ⓘ Repeat the amplification reaction and add more DNA.
c) Problems with DNA	ⓘ If necessary, repeat the DNA purification. Repeat the amplification reaction.
d) Thermal cycler incorrectly programmed	ⓘ Check to see if the correct cycling conditions were used. If necessary, calibrate the thermal cycler.
False-positive signals	
a) Temperature during hybridization and the stringent wash too low	ⓘ Nonspecific signals can occur if the temperature for hybridization and the stringent wash is too low. The temperature should be 50 ± 0.5°C. Make sure to set the waterbath correctly. Always close the lid of the waterbath during incubation.
b) Too much DNA used	ⓘ Check the PCR by agarose gel electrophoresis. If too much DNA was added, a smear of DNA is often visible on the gel. Due to the low annealing temperature of the PCR some background bands on the gel are normal. Dilute the DNA and repeat the amplification by adding 10 times less DNA.

	Comments and suggestions
c) Contamination	ⓘ If a similar nonspecific pattern occurs on most of the strips, contamination may be present. Contamination may be introduced during handling of clinical samples, DNA purification, or PCR setup. Therefore, repeat the amplification reaction using freshly prepared solutions and/or repeat the DNA purification procedure. If the negative DNA control is negative, but the samples remain positive, it is most likely that contamination has occurred during handling of the clinical samples.
d) **Manual procedure**: Hybridization solution (HS) or Stringent Wash (SW) is not properly heated or mixed	ⓘ Before use, warm Hybridization Solution (HS) and Stringent Wash (SW) to at least 37°C in a waterbath. Make sure that the temperature does not exceed 50°C. All crystals should be dissolved before use.
Decoloration or white stain at the center of the strip or uneven staining	
a) **Manual procedure**: Conjugate incorrectly diluted or conjugate excess not properly washed away	ⓘ Repeat the color development procedure using the same strips. Increase the speed and make sure the strips are completely submerged in reagent.
High background color	
a) **Manual procedure**: Conjugate incorrectly diluted or conjugate excess not properly washed away	ⓘ Repeat the test with new strips starting with the denaturation step (Step 7 in Protocol 4: Detection of Amplified HPV DNA Using an Automated Procedure or step 1 in Protocol 1: Detection of Amplified HPV DNA Using a Manual Procedure — Hybridization). Use the same amplified material. Make sure the strips move back and forth in the troughs during the wash steps with 1x rinse solution in the color development procedure. Wash the strips for at least 1 min.

	Comments and suggestions
b) **Manual procedure**: Strips incubated in substrate solution for too long	ⓘ Repeat the test with new strips, starting with the denaturation step (Step 7 in automated protocol or step 1 in Protocol 1: Detection of Amplified HPV DNA Using a Manual Procedure — Hybridization). Use the same amplified material. Make sure the strips move back and forth in the troughs during the wash steps with 1x rinse solution in the color development procedure. Wash the strips for at least 1 min.
c) **Automated procedure**: Insufficient buffer prepared	ⓘ Make sure to prepare sufficient buffer. See "Preparing reagents for the automated procedure," page 22, for more information.
d) **Automated procedure**: Tubes that disperse the buffers not placed correctly into vessels containing	ⓘ Be sure to place tubes correctly into the vessels containing buffers.
e) **Automated procedure**: Strips left for too long in 1x rinse solution	ⓘ Strips can be left in the instrument no longer than overnight. Repeat the test with new strips. Use the same amplified material.
Weak signals for the beta-globin probe line	
The PCR was designed to optimally detect HPV DNA.	ⓘ It is possible that for some DNA peparation procedures or sample types, the beta-globin signal will always be weak or absent and the samples will not necessarily be invalid. Please contact technical support.

Appendix A: Interpretation of Results

Positive result

The presence of a visible line is considered a positive reaction. The interpretation of the test result is directly linked to the probe name of the HPV type (for example, a purple color on probe line HPV16 indicates the presence of HPV type 16). Multiple HPV types can be present in a single sample.

Quality control

The first positive line is the conjugate control line (immediately below the marker line). This line provides a control for the addition of reactive conjugate and substrate solution during the detection procedure. It must always be positive and should have approximately the same intensity on each strip in the same test run. If the control band is not visible, the run is invalid. The last probe line is used as the internal control. It reacts with the co-amplified human genomic DNA present in the clinical sample. It is used as an internal control to check for PCR inhibition and adequate sample taking and/or DNA isolation. If HPV is present in the sample the beta-globin probe line can be negative due to PCR competition as the HPV DNA is preferentially amplified.

Table 15. Result analysis

Conjugate Control	HPV Result	beta-Globin	Result	Comment
+	+	+	valid	
+	+	–	valid	HPV DNA is preferentially amplified, resulting in an absent beta-globin signal (PCR competition)
+	–	+	valid	
-	+/-	+/-	invalid	Repeat detection assay with the same amplimer

Table continued on next page.

Table 15. Continued

Conjugate Control	HPV Result	beta-Globin	Result	Comment
+	–	–	invalid	Either the DNA purification or the PCR failed. Repeat the entire assay starting with DNA purification through PCR and reverse hybridization using the original sample. If the same result is obtained, contact technical support.

Appendix B: Control of Contamination in PCRs

It is extremely important to include at least one negative control that lacks the template nucleic acid in every PCR setup to detect possible contamination. For the negative control, only the conjugate control line should be positive.

Recommendations for laboratory design and procedures

The following sequence of operations is recommended:

1. Preparation and aliquoting of PCR mixes
2. Preparation of samples (DNA isolation)
3. Amplification
4. Analysis of the biotinylated PCR products by reverse hybridization

Personnel involved in steps 3 and 4 should not participate in subsequent work for steps 1 and 2 on the same day. Similarly, after being involved in step 2, personnel should not participate in subsequent work for step 1 on the same day.

To prevent contamination (for example, with amplification products) of specimens and to avoid false-positive results, the procedure should be performed in three physically separated rooms, each with its own set of supplies and pipets. One room is needed for reagent preparation, another for sample preparation, and a third room for amplification and PCR product detection. All equipment should be kept in the room where it is used and should not be transferred between rooms. Filter tips should be used for pipetting to minimize cross-contamination between specimens. In addition, wear disposable gloves and change them frequently.

Room 1: This room should be used only for storage and preparation of PCR reagents. This room and its equipment must be kept free of amplification products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room. Disposable gloves should be worn at all times.

Room 2: This room is used for sample preparation and must be kept free of amplification products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room. During sample preparation, disposable gloves should be changed frequently. Carefully uncap vials containing processed sample to avoid cross-contamination. Avoid opening more than one reaction vial containing sample at the same time.

Room 3: This room is used for amplification and detection of PCR products. Laboratory personnel should wear a clean laboratory coat, which must not be worn outside this room and must be changed daily. When working with amplification products, disposable gloves should be worn.

The *digene* HC2 High-Risk HPV Test and the *digene* Genotyping RH Test, Detection Kit can be performed in the same room. When doing so, perform the specimen processing, denaturation, and transfer to the hybridization plate for the HC2 test prior to entering the HC2 and Genotyping RH Detection testing laboratory (Room 3). This prevents the original specimen, which should be processed in Room 2, from exposure to amplification products used in Room 3.

In case of contamination, laboratory benches, apparatus, and pipets can be decontaminated by cleaning them with DNA AWAY and RNase AWAY (Molecular BioProducts) or a 1/10 dilution of a commercial bleach solution.*† Afterwards, the benches and pipets should be rinsed with RNase-free water.

General chemical precautions

We recommend storing PCR stock solutions in small aliquots and using fresh aliquots for each PCR.

References

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Cited reference

1. van den Brule, A. J., Pol R., Fransen-Daalmeije, N., Schouls, L. M., Meijer, C. J., and Snijders, P. J. (2002) GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J Clin Microbiol **40**, 779.

* Most commercial bleach solutions are approximately 5.25% sodium hypochlorite. Sodium hypochlorite is an irritant and should be handled with caution.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

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Ordering Information

Product	Contents	Cat. no.
digene HPV Genotyping RH Test (20)	*digene* HPV Genotyping RH Test, Amplification Kit, *digene* HPV Genotyping RH Test, Detection Kit	613413
Related products		
QIAamp MinElute Virus Spin Kit (50)	For 50 minipreps: 50 QIAamp MinElute Columns, QIAGEN Protease, Carrier RNA, Buffers, Collection Tubes (2 ml)	57704
EZ1® DSP Virus Kit (48)	For 48 viral nucleic acid preps: Pre-filled Reagent Cartridges, Disposable Filter-Tips, Disposable Tip Holders, Sample Tubes, Elution Tubes, Buffers, Carrier RNA	62724

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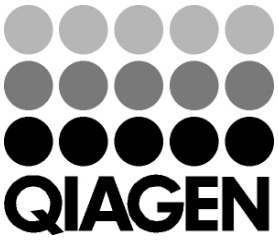

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