January 2010

SeqTarget Normalization Handbook

For purification and normalization of
SeqTarget long-range PCR fragments
(>2 kb)

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

SeqTarget Normalization Kit	(480)
Catalog no.	**122217**
Number of preps	**480**
QIAquick® 96 Plates	5
Bead Filter 96 Plates	5
Buffer PBN1	50 ml
Buffer PBN2	25 ml
Buffer EB	55 ml
Buffer EB2	25 ml
Normalization Beads	25 ml
Mineral Oil	50 ml
Handbook	1

Storage

QIAquick 96 plates and Bead Filter 96 plates should be stored dry at room temperature (15–25°C). Under these conditions, QIAquick 96 plates and Bead Filter 96 plates can be stored for up to 12 months without showing any reduction in quality or performance.

Buffers should be stored at temperatures indicated on the bottles. Normalization Beads and Buffer PBN1 should be stored at 2–8°C and should never be frozen.

When stored under these conditions and handled correctly, this kit can be stored at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

All buffers and plates should be at room temperature when used.

Product Use Limitations

The SeqTarget Normalization Kit is intended for molecular biology applications. This product is neither intended for the diagnosis, prevention, or treatment of a disease, nor has it been validated for such use either alone or in combination with other products. Therefore, the performance characteristics of the products for clinical use (i.e., diagnostic, prognostic, therapeutic, or blood banking) are unknown.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

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 SeqTarget Normalization 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.

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

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the SeqTarget Normalization Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Recent technological advances in sequencing systems have rapidly increased sequencing capacity and sequence output. However, these second-generation sequencing systems do not currently have the throughput capacity to sequence the whole human genome cost-effectively. Therefore, a reduction in the complexity of genomic DNA samples to a manageable subset is required prior to sequencing.

The SeqTarget system has been developed to overcome this limitation and enable long-range PCR amplification, PCR product purification, and normalization of target genomic DNA regions to meet second-generation resequencing throughput requirements.

The SeqTarget system consists of 3 components:

- SeqTarget Primer Select (cat. no. 122001) and SeqTarget Primer Select 96 Plate (cat. no. 122004)
- SeqTarget LongRange PCR Kit (500) (cat. no. 122117)
- SeqTarget Normalization Kit (480) (cat. no. 122217)

SeqTarget Primer Select

SeqTarget Primer Select is a tool that enables primer design and convenient ordering at QIAGEN's GeneGlobe® Web portal (www.qiagen.com/goto/GeneGlobe). This innovative tool ensures optimal design and ordering of bioinformatically-evaluated, unique primers for overlapping long-range PCR fragments of human genes. Binding of primers to annotated repeat regions is avoided and special requirements for successful amplification of fragments of approximately 10 kb are taken into consideration.

SeqTarget LongRange PCR Kit

The SeqTarget LongRange PCR Kit contains an enzyme blend of thermostable DNA polymerases with enhanced processivity and proofreading ability. This ensures both a very high extension rate as well as increased fidelity, enabling reliable amplification of genomic targets longer than 20 kb.

SeqTarget Normalization Kit

The SeqTarget Normalization Kit enables isolation of long-range PCR fragments by size-selective purification. The first step involves removal of unincorporated primers and nucleotides and reduction of PCR background (removal of fragments ≤2 kb). Subsequently, PCR fragments are normalized (i.e. brought to comparable amounts) by binding to beads with limited binding capacity. Excess PCR products are simply washed off and normalized amounts of PCR products are eluted with a mild buffer at a slightly alkaline pH. Normalized PCR products of similar size can be pooled together as they are equimolar. No further quantification and normalization steps are necessary for library preparation. Subsequent sequencing can be easily performed without introducing any bias. In addition, expensive detection devices for A_{260}/A_{280} or fluorescence measurements are not required and further adjustment to obtain the desired concentration is not needed.

Principle and procedure

The SeqTarget procedure consists of 2 steps: purification and subsequent normalization.

Purification

Long-range PCR fragments are purified by size exclusion and adsorbed onto a silica membrane. Following long-range PCR amplification using the SeqTarget LongRange PCR Kit, the optimized binding buffer, Buffer PBN1, is used to reduce PCR background by removing unincorporated primers and nucleotides as well as DNA fragments that are ≤2 kb in size. Impurities and small DNA fragments are efficiently removed in a wash step with 70% ethanol and pure DNA is eluted using Buffer EB for subsequent normalization (Figure 1).

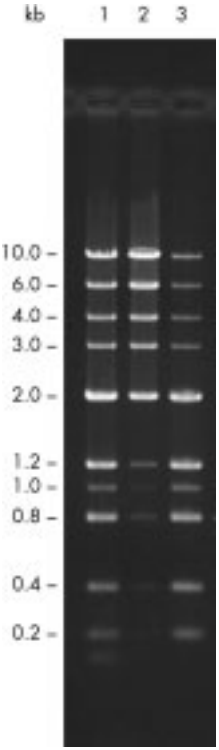

Figure 1. Successful purification of long-range PCR fragments using the SeqTarget Normalization Kit. To simulate background reduction during purification of long-range PCR fragments, a DNA molecular weight marker ranging from 100 bp to 10 kb was purified by size-selective purification using the SeqTarget Normalization Kit. DNA fragments of up to 2 kb were removed or reduced by the purification procedure, respectively. **Lane 1:** Unpurified molecular weight marker. **Lane 2:** Eluate after purification. **Lane 3:** Flow-through.

Normalization

The procedure involves binding of DNA to positively-charged magnetic beads with limited binding capacity under acidic conditions that are established with Buffer PBN2. PCR products are then transferred to the Bead Filter 96 plate, which has a low DNA-binding capacity. Excess PCR products are removed by a wash step using deionized water. Subsequently, normalized amounts of PCR products are eluted with Buffer EB2 (50 mM Tris·HCl, pH 8.5). PCR products of similar size are equimolar and can therefore be pooled without the need for further quantification and normalization steps for library generation (e.g., shotgun library approach). Subsequent sequencing can be performed without bias.

DNA yield

The DNA yield can be adjusted by modification of the bead amount (Figure 2). Following the purification procedure, typically 50 µl Normalization Beads bind approximately 500 ng DNA (±50%) from a sample containing at least from 3-fold (>1.5 µg) to 10-fold (~5 µg) excess DNA.

Note: It is recommended to analyze large amplification products on a suitable agarose gel (0.7–1%), preferably in Buffer TAE, using appropriate DNA markers to determine correct fragment size. For reliable agarose gel analysis of original long-range PCR products, a 0.5–1 µl sample (1/50 to 1/25 volume of a 25 µl PCR reaction) should be sufficient. For normalized PCR fragments, 3 µl of the final Buffer EB2 eluate should give clear, homogeneous bands. Also, following the purification step, the eluate can be analyzed on agarose gel. A 5–10 µl volume of eluate in Buffer EB should be adequate for analysis.

Note: If required, the yield of the normalized DNA can be determined by OD measurement using a spectrophotometer or by fluorescent dye staining.

For determination of the DNA concentration after the purification procedure by A_{260} measurement, Buffer EB should be used as a blank. For determination of the concentration of the normalized DNA by A_{260} measurement, Buffer EB2 should be used as a blank.

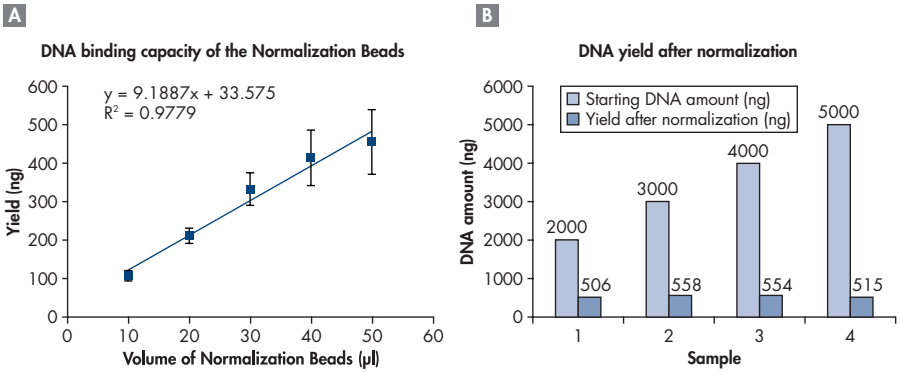

Figure 2. Typical example of the DNA-binding capacity of the Normalization Beads. A DNA-binding capacity of different bead quantities was determined for 4 different SeqTarget LongRange PCR fragments. B Example of normalization of different amounts of a DNA sample ranging from 2 µg to 5 µg using a 50 µl bead suspension. The average yield of normalized DNA was 533 ng. Normalized DNA was quantified using the Quant-iT™ dsDNA Broad-Range Assay Kit (Invitrogen).

Description of protocols

Three protocols have been specially developed and optimized to meet different throughput requirements and to allow the use of different laboratory equipment.

Using a microtiter plate centrifuge and vacuum manifold

For manual handling or partial automation, follow "Protocol 1: SeqTarget Purification and Normalization Using a Laboratory Microtiter Plate Centrifuge and Vacuum Manifold" on pages 15–18. Highest accuracy and reproducibility is achieved using this protocol. The protocol is for centrifugation and vacuum filtration procedures and is less time consuming than Protocol 2.

Using a vacuum manifold

"Protocol 2: SeqTarget Purification and Normalization Using a Vacuum Manifold" (pages 19–22) can be easily adapted to different robotic systems equipped with plate-handling systems and a vacuum manifold station. Only the final elution step requires centrifugation. The accuracy and reproducibility achieved through this protocol is comparable to Protocol 1.

Using a laboratory microtiter plate centrifuge

"Protocol 3: SeqTarget Purification and Normalization Using a Laboratory Microtiter Plate Centrifuge" (pages 23–26) does not require the use of a vacuum manifold or robotic station. The protocol is specially developed for quick manual processing using a laboratory microtiter plate centrifuge. Following normalization, there might be a slight variation in the DNA yield compared to protocols 1 and 2 and the binding capacities stated for the kit can only be used as a guideline. Therefore, recalibration of the binding capacity of the beads for exact adjustment of desired values is recommended.

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.

Equipment

- QIAvac 96 (cat. no. 19504)
- Vacuum Regulator (cat. no. 19530)
- Centrifuges: We recommend QIAGEN's Centrifuge 4-16 (cat. no. 80310) for room-temperature centrifugation, and for refrigerated centrifugation, we recommend QIAGEN's Centrifuge 4-16K (cat. no. 81401).
- Plate Rotor (2 x 96) (cat. no. 81031)
- Eppendorf® Thermomixer Comfort/Eppendorf Thermomixer Compact or other suitable shakers for microplates and deep-well blocks

Consumables

- S-Blocks (24) (cat. no. 19585)
- 96-Well Microplates MP (20) (1031656)

Reagents

- Deionized water
- 70% ethanol: To prepare 500 ml 70% ethanol, mix 370 ml 95% ethanol with 130 ml deionized water.

Important Notes

Guidelines for QIAvac manifolds

The following recommendations should be followed when handling QIAvac manifolds.

- QIAvac manifolds operate with a house vacuum or Vacuum Pump (e.g., Vacuum Pump, cat. no. 84010 [USA and Canada], 84000 [Japan], or 84020 [rest of the world]).
- Always store QIAvac manifolds clean and dry. To clean, simply rinse all components with water and dry with paper towels. Do not air dry, as the screws may rust and need to be replaced. Do not use abrasives or solvents.
- Always place the QIAvac manifold on a secure bench top or work area. If dropped, the manifold may crack.
- If solvents are spilled on the unit, rinse thoroughly with distilled water. Ensure that no residual buffer remains in the vacuum manifold.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of a QIAvac manifold.

Vacuum notes

- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during manipulations.
- Wear safety glasses when working near a manifold under pressure.
- For safety reasons, do not use 96-well plates that have been damaged in any way.
- The vacuum pressure is the pressure difference between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be measured using a vacuum regulator (see ordering information, page 30). Table 1 provides pressure conversions to other units.
- Use of a vacuum pressure lower than recommended may reduce DNA yield and purity.

Table 1. Pressure conversions

Ton convert from millibars (mbar) to	Multiply by
Millimeters of mercury (mm Hg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0295
Torrs (Torr)	0.75
Atmospheres (atm)	0.000987
Pounds per square inch (psi) 0.0145	0.0145

Centrifugation notes

All centrifugation steps are carried out as outlined in the protocols in a conventional, microplate centrifuge.

We recommend QIAGEN's Centrifuge 4-16 (cat. no. 80310) or QIAGEN's Centrifuge 4-16K (cat. no. 81401) with a Plate Rotor (2 x 96) (cat. no. 81031).

If using a centrifuge from other vendors, a conversion of rpm to *g*-force and vice versa may be necessary.

rpm *g*-force calculator

$rcf = 0.00001118 * r * S^2$

$$S = \sqrt{\frac{rcf}{0.00001118 * r}}$$

rcf is the relative centrifugal force in *g*

r is the rotating radius in cm

S is the speed of the centrifuge in revolutions per minute (rpm)

Protocol 1: SeqTarget Purification and Normalization Procedure

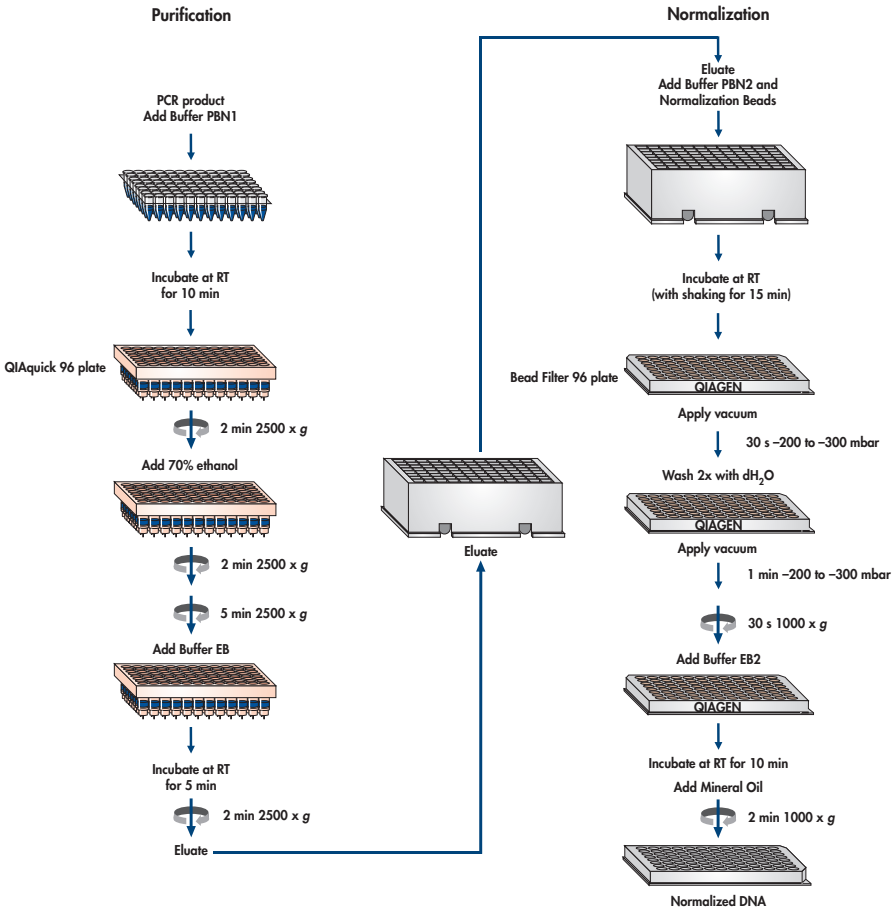

Figure 3. SeqTarget purification and normalization using a laboratory microtiter plate centrifuge and vacuum manifold.

Protocol 1: SeqTarget Purification and Normalization Using a Laboratory Microtiter Plate Centrifuge and Vacuum Manifold

This protocol is optimized for the purification and normalization of long-range PCR products of the highest achievable quality and reproducibility (Figure 3, page 15).

Important points before starting

- Please read "Important Notes" (pages 13–14) carefully.
- All protocol steps should be carried out at room temperature (15–25°C).

Purification procedure

1. **Transfer 10–20 µl PCR product (obtained using the SeqTarget LongRange PCR Kit) to a new microplate. Add exactly 2 volumes of Buffer PBN1 to 1 volume of the PCR reaction and mix by pipetting up and down 6 times. Incubate for 10 min.**

   IMPORTANT: The chosen volume of the long-range PCR product should contain at least a 3-fold excess of DNA. For example, if the designated amount of normalized DNA should be 500 ng, at least 1.5 µg high-quality PCR product (with minimal background) is required. For samples with an increased level of background, the amount of DNA should be increased accordingly (to more than a 3-fold excess).

   Note: Pipetting accuracy is critical. Buffer PBN1 is viscous and care should therefore be taken when pipetting to ensure that the correct volume of buffer is added. Pipetting and dispensing Buffer PBN1 too quickly may result in an inaccurate volume. Dipping the pipette tip too deep may result in accidental transfer of droplets present on the outside of the tip, leading to the delivery of an inaccurate volume of buffer. An inaccurate DNA:buffer ratio may influence the yield and size exclusion of the purified DNA.

   Note: Do not centrifuge samples after addition of Buffer PBN1.

2. **Place a QIAquick 96 plate on top of a waste block (S-Block) and pipet the samples from step 1 into the wells of the QIAquick 96 plate. Centrifuge the plate at 2500 x *g* for 2 min in a bench-top centrifuge with a rotor for microtiter plates (e.g., QIAGEN's Centrifuge 4-16 or 4-16K). Discard the flow-through. Place the QIAquick 96 plate back onto the waste block.**

   Note: The waste block may be reused for different samples and experiments.

3. **To wash, add 750 µl 70% ethanol into each well of the QIAquick 96 plate and centrifuge at 2500 x *g* for 2 min.**

4. **Discard the flow-through and place the plate back onto the waste block. Centrifuge at 2500 x *g* for an additional 5 min.**

   IMPORTANT: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.

5. **Place a QIAquick 96 plate on top of a clean S-Block.**

6. **To elute DNA, add 100 µl Buffer EB (10 mM Tris·HCl, pH 8.5) to each well of the QIAquick 96 plate, incubate for 5 min, and then centrifuge for 2 min at 2500 x *g*.**

Normalization procedure

7. **Add 50 µl Buffer PBN2 and an appropriate volume of Normalization Beads (e.g., 50 µl for binding of 500 ng DNA) to the eluate from step 6 of the purification procedure. Mix by pipetting up and down 10 times or by vigorous horizontal pulse shaking. Do not vortex as this can cause spraying of beads. Incubate with shaking to keep the suspension homogenous (e.g., at 1000 rpm when using an Eppendorf Thermomixer) for 15 min.**

   Note: Since the bead amount is critical for the yield of normalized DNA, ensure that the Normalization Beads are homogenized immediately before dispensing them. To prevent sedimentation of the beads, resuspend the beads by vigorous shaking periodically and immediately before dispensing the beads into the samples.

   Note: Adjust the desired amount of normalized DNA by adjusting the volume of the Normalization Beads according to the binding capacity (see Figure 2A, page 10). **The maximum volume of Normalization Beads should not exceed 50 µl.**

   Do not centrifuge suspensions containing Normalization Beads.

   Optional: Buffer PBN2 and the Normalization Beads can be premixed before adding to the samples. This mixture has to be used within 2 hours and cannot be stored for longer periods. Resuspend the beads by vigorous shaking before adding to the samples.

8. **During incubation, prepare the QIAvac 96 (see pages 13–14).**
   - Place the waste tray inside the QIAvac base.
   - Place the QIAvac 96 top plate squarely over the base.
   - Place the Bead Filter 96 plate in the QIAvac 96 top plate, making sure that the plate is placed securely. Seal unused wells of the filter plate with tape.
   - Attach the QIAvac 96 to a vacuum source.

9. **Transfer the suspension from step 7 to the Bead Filter 96 plate and apply a vacuum of –200 to –300 mbar for 30 s.**

   Note: After the solution has completely passed through the filter, switch off the vacuum. In case the solution has not passed through the filter completely, extend the filtration time. Do not apply a vacuum pressure lower than –300 mbar.

10. **Add 300 µl deionized water and switch on the vacuum source for 1 min at between –200 to –300 mbar.**

    Note: DNA not bound to the beads will be washed away. Ensure that the water has passed through the filter completely.

11. **Repeat step 10.**

12. **Place the Bead Filter 96 plate on top of a waste block and centrifuge for 30 s at 1000 x *g*.**

    Note: This step is necessary to remove any residual wash water.

13. **Place the Bead Filter 96 plate on top of a clean 96-well microplate and add 20 µl Buffer EB2 making sure that there is no contact between the pipet tip and the filter. Incubate at room temperature for 10 min.**

14. **Add 100 µl mineral oil to each well containing Buffer EB2 and centrifuge for 2 min at 1000 x *g* to elute the normalized DNA.**

    Note: Mineral oil is required for complete elution of the normalized DNA. Mineral oil can pass through a dry membrane. However, it does not pass through a wet filter membrane during centrifugation. This ensures that there is no carry-over contamination with mineral oil.

    Note: The volume of the eluted DNA should be approximately 20 µl. If the aqueous phase has not passed through the filter completely, extend the centrifugation time. **Do not increase the centrifugation force.**

    Note: After purification and normalization, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

Protocol 2: SeqTarget Purification and Normalization Procedure

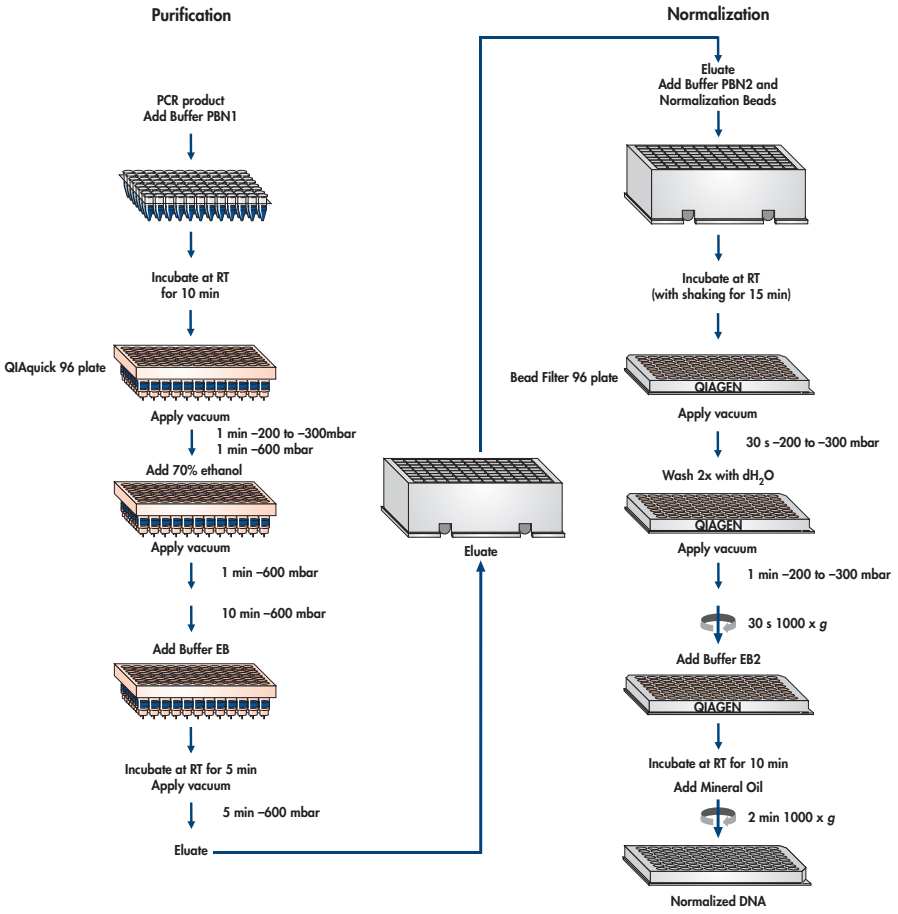

Figure 4. SeqTarget purification and normalization using a vacuum manifold.

Protocol 2: SeqTarget Purification and Normalization Using a Vacuum Manifold

This protocol is optimized for the purification and normalization of long-range PCR products using a vacuum manifold (Figure 4, page 19).

Important points before starting

- Please read "Important Notes" (pages 13–14) carefully.
- All protocol steps should be carried out at room temperature (15–25°C).

Purification procedure

1. **Transfer 10–20 µl PCR product (obtained using the SeqTarget LongRange PCR Kit) to a new microplate. Add exactly 2 volumes of Buffer PBN1 to 1 volume of the PCR reaction and mix by pipetting up and down 6 times. Incubate for 10 min.**

   IMPORTANT: The chosen volume of the long-range PCR product should contain at least a 3-fold excess of DNA. For example, if the designated amount of normalized DNA should be 500 ng, at least 1.5 µg high-quality PCR product (with minimal background) is required. For samples with an increased level of background, the amount of DNA should be increased accordingly (to more than a 3-fold excess).

   Note: Pipetting accuracy is critical. Buffer PBN1 is viscous and care should therefore be taken when pipetting to ensure that the correct volume of buffer is added. Pipetting and dispensing Buffer PBN1 too quickly may result in an inaccurate volume. Dipping the pipette tip too deep may result in accidental transfer of droplets present on the outside of the tip, leading to the delivery of an inaccurate volume of buffer. An inaccurate DNA:buffer ratio may influence the yield and size exclusion of the purified DNA.

   Note: Do not centrifuge samples after addition of Buffer PBN1.

2. **During incubation, prepare the QIAvac 96 (see pages 13–14).**
   - Place the waste tray inside the QIAvac base.
   - Place the QIAvac 96 top plate squarely over the base.
   - Place the QIAquick 96 plate in the QIAvac 96 top plate, making sure that the plate is placed securely. Seal unused wells of the plate with tape.
   - Attach the QIAvac 96 to a vacuum source.
3. **Pipet the samples from step 1 into the wells of a QIAquick 96 plate. Apply a vacuum of –200 to –300 mbar for 1 min. Increase the vacuum to –600 mbar for an additional 1 min.**
4. **To wash, add 750 µl 70% ethanol into each well of the QIAquick 96 plate and apply a vacuum of –600 mbar for 1 min.**

5. **Switch off the vacuum source and ventilate the QIAvac manifold. Tap the QIAquick 96 plate on a stack of absorbent paper to remove remaining droplets and place the plate back into the manifold. Apply maximum vacuum for an additional 10 min to dry the membrane.**
6. **Replace the waste tray with the S-Block. Place the QIAquick 96 plate back on the base.**
7. **To elute DNA, add 120 µl Buffer EB (10 mM Tris·HCl, pH 8.5) to the membrane, incubate for 5 min, and apply a vacuum of –600 mbar for 5 min.**

   Note: The eluted DNA should have a volume of 90–95 µl.

Normalization procedure

8. **Add 50 µl Buffer PBN2 and an appropriate volume of Normalization Beads (e.g., 50 µl for binding of 500 ng DNA) to the eluate from step 6 of the purification procedure. Mix by pipetting up and down 10 times or by vigorous horizontal pulse shaking. Do not vortex as this can cause spraying of beads. Incubate with shaking to keep the suspension homogenous (e.g., at 1000 rpm when using an Eppendorf Thermomixer) for 15 min.**

   Note: Since the bead amount is critical for the yield of normalized DNA, ensure that the Normalization Beads are homogenized immediately before dispensing them. To prevent sedimentation of the beads, resuspend the beads by vigorous shaking periodically and immediately before dispensing the beads into the samples.

   Note: Adjust the desired amount of normalized DNA by adjusting the volume of the Normalization Beads according to the binding capacity (see Figure 2A, page 10). **The maximum volume of Normalization Beads should not exceed 50 µl.**

   Do not centrifuge suspensions containing Normalization Beads.

   Optional: Buffer PBN2 and the Normalization Beads can be premixed before adding to the samples. This mixture has to be used within 2 hours and cannot be stored for longer periods. Resuspend the beads by vigorous shaking before adding to the samples.

9. **During incubation prepare the QIAvac 96 (see pages 13–14).**
   - Place the waste tray inside the QIAvac base.
   - Place the QIAvac 96 top plate squarely over the base.
   - Place the Bead Filter 96 plate in the QIAvac 96 top plate, making sure that the plate is placed securely. Seal unused wells of the filter plate with tape.
   - Attach the QIAvac 96 to a vacuum source.

10. **Transfer the suspension from step 8 to the Bead Filter 96 plate and apply a vacuum of –200 to –300 mbar for 30 s.**

    Note: After the solution has completely passed through the filter, switch off the vacuum. In case the solution has not passed through the filter completely, extend the filtration time. Do not apply a vacuum pressure lower than –300 mbar.

11. **Add 300 µl deionized water and switch on the vacuum source for 1 min at between –200 to –300 mbar.**

    Note: DNA not bound to the beads will be washed away. Ensure that the water has passed through the filter completely.

12. **Repeat step 11.**

13. **Place the Bead Filter 96 plate on top of a waste block and centrifuge for 30 s at 1000 x *g*.**

    Note: This step is necessary to remove any residual wash water.

14. **Place the Bead Filter 96 plate on top of a clean 96-well microplate and add 20 µl Buffer EB2, making sure that there is no contact between the pipet tip and the filter. Incubate at room temperature for 10 min.**

15. **Add 100 µl mineral oil to each well containing Buffer EB2 and centrifuge for 2 min at 1000 x *g* to elute the normalized DNA.**

    Note: Mineral oil is required for complete elution of the normalized DNA. Mineral oil can pass through a dry membrane. However, it does not pass through a wet filter membrane during centrifugation. This ensures that there is no carry-over contamination with mineral oil.

    Note: The volume of the eluted DNA should be approximately 20 µl. If the aqueous phase has not passed through the filter completely, extend the centrifugation time. **Do not increase the centrifugation force.**

    Note: After purification and normalization, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

Protocol 3: SeqTarget Purification and Normalization Procedure

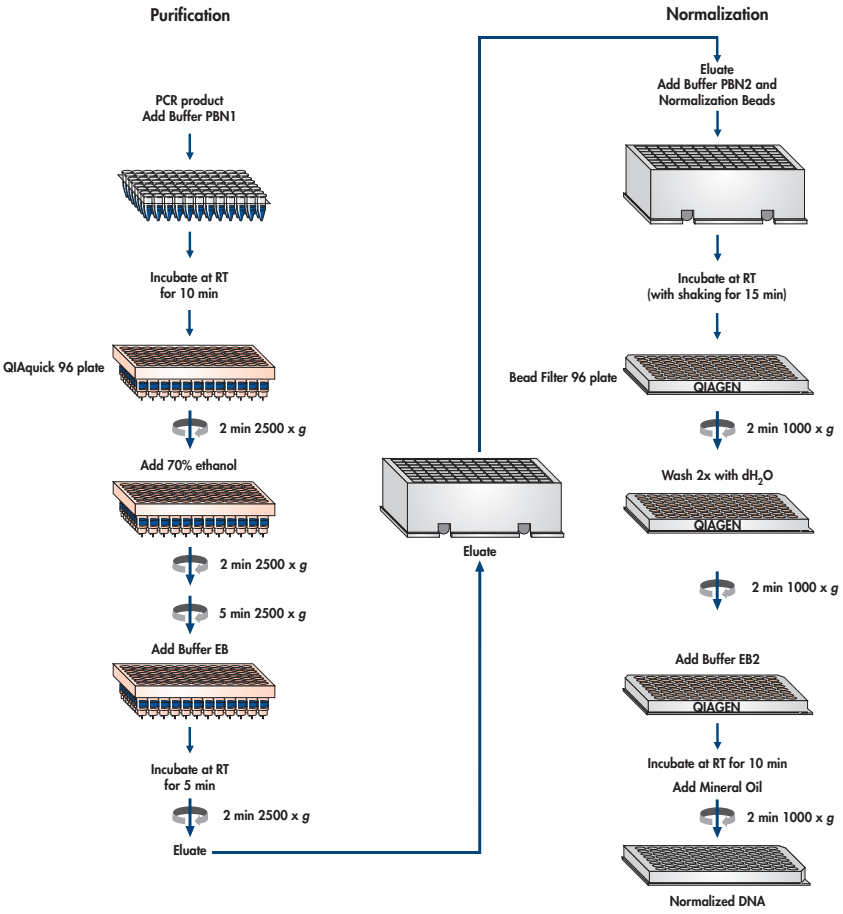

Figure 5. SeqTarget purification and normalization using a microtiter plate centrifuge.

Protocol 3: SeqTarget Purification and Normalization Using a Microtiter Plate Centrifuge

This protocol is optimized for the purification and normalization of long-range PCR products using a microtiter plate centrifuge (Figure 5, page 23).

Important points before starting

- Please read "Important Notes" (pages 13–14) carefully.
- All protocol steps should be carried out at room temperature (15–25°C).

Purification procedure

1. **Transfer 10–20 µl PCR product (obtained using the SeqTarget LongRange PCR Kit) to a new microplate. Add exactly 2 volumes of Buffer PBN1 to 1 volume of the PCR reaction and mix by pipetting up and down 6 times. Incubate for 10 min.**

   IMPORTANT: The chosen volume of the PCR product should contain at least a 3-fold excess of DNA. For example, if the designated amount of normalized DNA should be 500 ng, at least 1.5 µg high-quality PCR product (with minimal background) is required. For samples with an increased level of background, the amount of DNA should be increased accordingly (to more than a 3-fold excess).

   Note: Pipetting accuracy is critical. Buffer PBN1 is viscous and care should therefore be taken when pipetting to ensure that the correct volume of buffer is added. Pipetting and dispensing Buffer PBN1 too quickly may result in an inaccurate volume. Dipping the pipette tip too deep may result in accidental transfer of droplets present on the outside of the tip, leading to the delivery of an inaccurate volume of buffer. An inaccurate DNA:buffer ratio may influence the yield and size exclusion of the purified DNA.

   Note: Do not centrifuge samples after addition of Buffer PBN1.

2. **Place a QIAquick 96 plate on top of a waste block (S-Block) and pipet the samples from step 1 into the wells of the QIAquick 96 plate. Centrifuge the plate at 2500 x *g* for 2 min in a bench-top centrifuge with a rotor for microtiter plates (e.g., QIAGEN's Centrifuge 4-16 or 4-16K). Discard the flow-through. Place the QIAquick 96 plate back onto the waste block.**

   Note: The waste block may be reused for different samples and experiments.

3. **To wash, add 750 µl 70% ethanol into each well of the QIAquick 96 plate and centrifuge at 2500 x *g* for 2 min.**

4. **Discard the flow-through and place the plate back onto the waste block. Centrifuge at 2500 x *g* for an additional 5 min.**

   IMPORTANT: Residual ethanol will not be completely removed unless the flow-through is discarded before this additional centrifugation step.

5. **Place a QIAquick 96 plate on top of a clean S-Block.**
6. **To elute DNA, add 100 µl Buffer EB (10 mM Tris·HCl, pH 8.5) to each well of the QIAquick 96 plate, incubate for 5 min, and then centrifuge for 2 min at 2500 x *g*.**

Normalization procedure

7. **Add 50 µl Buffer PBN2 and an appropriate volume of Normalization Beads (e.g., 50 µl for binding of 500 ng DNA) to the eluate from step 6 of the purification procedure. Mix by pipetting up and down 10 times or by vigorous horizontal pulse shaking. Do not vortex as this can cause spraying of beads. Incubate with shaking to keep the suspension homogenous (e.g., at 1000 rpm when using an Eppendorf Thermomixer) for 15 min.**

   Note: Since the bead amount is critical for the yield of normalized DNA, ensure that the Normalization Beads are homogenized immediately before dispensing. To prevent sedimentation of the beads, resuspend the beads by vigorous shaking periodically and immediately before dispensing the beads into the samples.

   Note: Adjust the desired amount of normalized DNA by adjusting the volume of the Normalization Beads according to the binding capacity (see Figure 2A, page 10). **The maximum volume of Normalization Beads should not exceed 50 µl.**

   Do not centrifuge suspensions containing Normalization Beads.

   Optional: Buffer PBN2 and the Normalization Beads can be premixed before adding to the samples. This mixture has to be used within 2 hours and cannot be stored for longer periods. Resuspend the beads by vigorous shaking before adding to the samples.

8. **Place the Bead Filter 96 plate on top of a waste block (S-Block) and transfer the samples from step 7 into the wells of the Bead Filter 96 plate. Centrifuge at 1000 x *g* for 2 min in a bench-top centrifuge with a rotor for microtiter plates (e.g., QIAGEN's Centrifuge 4-16 or 4-16K). Discard the flow-through. Place the Bead Filter 96 plate back onto the waste block.**

   Note: The liquid should have passed the membrane completely. In case some liquid remains on top of the filter, extend the centrifugation for an additional 30 s. Do not increase the centrifugation force.

9. **Add 300 µl deionized water and centrifuge for 2 min at 1000 x *g*.**

   Note: DNA not bound to the beads will be washed away. Ensure that the water has passed through the filter completely. In case some liquid remains on top of the filter, extend the centrifugation for an additional 30 s. Do not increase the centrifugation force.

10. **Repeat step 9.**

11. **Place the Bead Filter 96 plate on top of a clean 96-well microplate and add 20 µl Buffer EB2, making sure that there is no contact between the pipet tip and the filter. Incubate at room temperature for 10 min.**

12. **Add 100 µl mineral oil to each well containing Buffer EB2 and centrifuge for 2 min at 1000 x *g* to elute the normalized DNA.**

    Note: Mineral oil is required for complete elution of the normalized DNA. Mineral oil can pass through a dry membrane. It does not, however, pass through a wet filter membrane during centrifugation. This ensures that there is no carry-over contamination with mineral oil.

    Note: The volume of the eluted DNA should be approximately 20 µl. If the aqueous phase has not passed through the filter completely, extend the centrifugation time. **Do not increase the centrifugation force.**

    Note: After purification and normalization, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

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
Low or no yield		
a)	Various factors	Low yields may be caused by a number of factors. To find the source of the problem, analyze fractions saved from each step in the procedure on an agarose gel.
b)	Weak PCR	Before purification, check DNA quantity and quality on an agarose gel. The DNA yield after the first purification step is strongly influenced by the quality of the PCR. High PCR background will result in a reduced yield of the specific fragment. Fragments of up to 2 kb will be removed by size-selective purification. The long-range PCR product of desired size (>2 kb) should be clearly visible on the agarose gel after loading 1/50 volume of the PCR reaction. In addition, after the purification step, the DNA quantity can be checked on an agarose gel by loading 5–10 µl (1/20–1/10 volume) after elution with Buffer EB.
c)	Loss of DNA during purification step	A decreased yield is observed if less than 2 volumes of Buffer PBN1 are added to the sample. Use the volumes specified in the protocols. After addition of Buffer PBN1, the pH should be acidic (pH ≤5.0). If this is not the case, check the storage conditions and expiry date of the buffers. The protocols in this handbook are optimized for PCR samples generated using the SeqTarget LongRange PCR Kit only.

	Comments and suggestions
No or reduced size selectivity during purification	
Buffer PBN1:DNA ratio	A Buffer PBN1:DNA ratio of >2:1 will reduce fragment size selectivity. Use the volumes specified in the protocols.
Strong variation in DNA yield after normalization	
a) DNA binding capacity of beads not reached	After the purification step, ensure that the DNA amount is in excess and is at least 3 times the binding capacity of the beads. Normalization of DNA with a high background (high amount of fragments ≤2 kb in size) will result in a lower yield of long-range PCR fragments of desired size. To determine if the beads were saturated with DNA, excess DNA can be precipitated using ethanol and checked on an agarose gel. After applying the sample to the Bead Filter 96 plate, the flow-through can be precipitated by adding 1/10 volume 3 M sodium acetate, pH 5.3 and 2.5 volumes ethanol, followed by centrifugation at maximum speed (13,000 rpm or ~17,000 x *g*) for 15 min in a bench-top centrifuge. The precipitated DNA can be dissolved in Buffer EB for subsequent loading on an agarose gel. Reduction of the bead amount or upscaling of the PCR reaction can prevent such problems associated with yield.
b) Wrong pH for binding	Ensure that 50 µl Buffer PBN2 is added to 100 µl DNA and Normalization beads. For DNA binding, the final pH has to be pH 5.0.
c) Incorrect amount of beads	Make sure that the Normalization Beads are homogenized immediately before dispensing. To prevent sedimentation of the beads, resuspend the beads by vigorous shaking periodically and immediately before dispensing to the samples. Do not use more than 50 µl Normalization Beads per DNA sample.

		Comments and suggestions
d)	DNA yield higher than expected	Bead Filter 96 plate was overloaded. Wash procedure was not sufficient to remove all the excess DNA, if more than 5 µg was loaded. Follow the recommendations from the protocol exactly.
e)	Clumping of beads	A mixture of beads and Buffer PBN2 tends to clump. Therefore, storage over extended periods should be avoided.
Beads in eluate		
	Bead carry over	In general, residual beads in the eluate after normalization do not interfere with subsequent applications. However, particles, if present, can be removed by magnetic separation (e.g., after pooling of the normalized PCR fragments).

Ordering Information

Product	Contents	Cat. no.
SeqTarget Normalization Kit (480)	For 480 preps: QIAquick 96 Plates, Bead Filter 96 Plates, Normalization Beads, Buffer PBN1, Buffer PBN2, Buffer EB, Buffer EB2, Mineral Oil	122217
SeqTarget Primer Select — for primer design and convenient ordering at GeneGlobe		
SeqTarget Primer Select	Tube format: 8.4 nmol SeqTarget Primer Select (containing a mix of lyophilized forward and reverse primers for a specific target); for 400 x 25 µl reactions	122001
SeqTarget Primer Select 96 Plate	96-well format: 8.4 nmol SeqTarget Primer Select (containing a mix of lyophilized forward and reverse primers for a specific target); for 400 x 25 µl reactions	122004
SeqTarget LongRange PCR Kit — for long-range PCR amplification, prior to normalization		
SeqTarget LongRange PCR Kit (500)	For 500 x 25 µl reactions: LongRange PCR Enzyme Mix (500 U), LongRange PCR Buffer, 5x Q-Solution®, RNase-Free Water, 10 mM dNTPs, and Control Primer Pair	122117
Accessories		
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder, Rack of Collection Microtubes (1.2 ml)	19504
Vacuum Regulator	For use with QIAvac manifolds	19530
S-Blocks (24)	96-well blocks with 2.2 ml wells, 24 per case	19585
96-Well Microplates MP (20)	96-well microplates, 20 per case	1031656

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