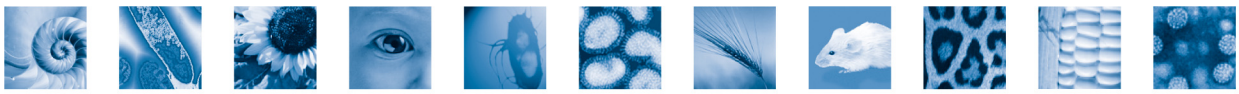
NimbleGen SeqCap EZ Exome Library SR User's Guide

Version 1. 2



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Not for use in diagnostic procedures.

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Chapter 1. Before You Begin

This *User's Guide* describes the process for the capture of genomic DNA (gDNA) from specified regions of the human genome using the SeqCap EZ Exome Library and amplification of captured DNA by ligation-mediated PCR (LM-PCR) (Figure 1). Specifically, this *User's Guide* describes a new Sequence Capture method that allows for the capture of DNA in solution rather than on arrays. This protocol starts with gDNA library prepared using the Illumina Paired-End Genomic DNA Sample Prep Kit. The captured human gDNA fragments can be sequenced directly using the Illumina Genome Analyzer II Instrument and reagents.

Thermocyclers should be programmed before beginning this protocol to the required thermocycler reaction programs detailed on page 16 (*Chapter 4*, Step 2.2), page 26 (*Chapter 7*, Step 2.2), and page 31 (*Chapter 8*, Table 1).

What's New?

- Modifications were made to the protocol for performing LM-PCR. Refer to page 15 (*Chapter 4*) and page 25 (*Chapter 7*).
- Modifications were made to the protocol for performing sample hybridization, including a 95% reduction in the amount of human COT DNA used as blocker in the hybridization reaction. Refer to page 19 (*Chapter 5*, Step 2.1) and page 20 (*Chapter 5*, Step 2.10).
- Instructions are provided for performing an optional quality control step to measure the quality of the amplified sample library before hybridization. Refer to page 39 (*Appendix B*).

To verify you are using the most up-to-date version of this *User's Guide* to process your arrays, go to *www.nimblegen.com/lit/*.

Terminology

LM-PCR: Linker Mediated PCR. In the context of this document, PCR using primers complementary to the sequencing adaptors.

Sequence Capture (or Capture): The process of enrichment of targeted regions from genomic DNA. In the context of this document, the hybridization of the amplified sample library and SeqCap EZ Exome Library and subsequent washing steps.

SeqCap EZ Exome Library (or Exome Library)**:** The complete set of biotinylated long oligonucleotide probes provided by Roche NimbleGen to perform sequence capture of the human exome (all coding exons).

Sample Library: The initial shotgun library generated from genomic DNA by fragmentation and ligation of sequencing-platform-specific linkers. In the context of this document, the sample library before amplification by LM-PCR and before capture.

Amplified Sample Library: The sample library after amplification by LM-PCR but before capture.

Captured DNA: The enriched DNA population from the amplified sample library after the capture process but before another round of LM-PCR.

Amplified Captured DNA: The captured DNA after LM-PCR amplification.

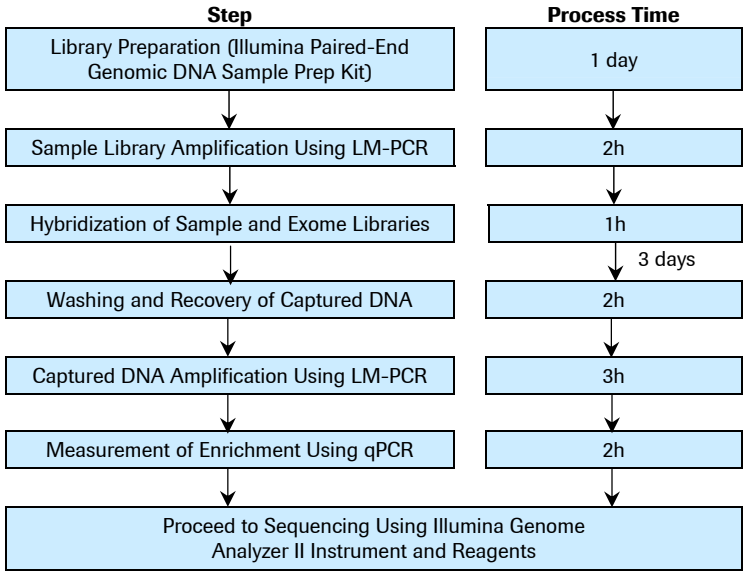

Figure 1: Workflow for SeqCap EZ Exome Library Experiments Using Illumina Genome Analyzer II Instrument. Steps in the procedure and estimated times for each step, based on the processing of one solution phase capture, are shown in the boxes. Incubation times are indicated beneath the appropriate process times.

Components Supplied

Component	Description
SeqCap EZ Exome Library (25 ng/µl)	4 reactions -or- 48 reactions
Product CD/DVD	Design (.gff[1]) and annotation (.bed[2]) files and user documentation are included.

1 View .gff (general feature format) files using Roche NimbleGen SignalMap software (demo version available at *www.nimblegen.com/products/software/*).

2 View .bed files using the Internet-based UCSC Genome browser.

Storage of the SeqCap EZ Exome Library

Aliquot and store the SeqCap EZ Exome Library as described in *Chapter 2* of this *User's Guide* (page 11).

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature unless indicated otherwise.

Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier	Catalog No.
DNA Vacuum Concentrator (1.5 ml tubes)	*Multiple Vendors*	
DynaMag-2 Magnet (16 x 1.5 ml tube holder)	Invitrogen	123-21D
Heat block	*Multiple Vendors*	
Water bath	*Multiple Vendors*	
Microcentrifuge with Multiplate Adaptors (12,000 x g capability)	*Multiple Vendors*	
Spectrophotometer	NanoDrop	ND-1000
Bioanalyzer 2100	Agilent	
LightCycler® 480 Instrument II	Roche Applied Science	05 015 243 001 (384-well) -or- 05 015 278 001 (96-well)
Thermocycler (capable of maintaining 47°C for 64 - 72 hours)	*Multiple Vendors*	
Vortex mixer	*Multiple Vendors*	

Consumables Available from Roche Applied Science

The package sizes listed provide sufficient material to perform a minimum of 10 Sequence Capture experiments.

Component	Package Size/Contents	Catalog No.
COT Human DNA, Fluorometric Grade	1 mg / ml, 1 ml	05 480 647 001
LightCycler® 480 Multiwell Plate 384 (with sealing foils)	5 x 10 plates	04 729 749 001
LightCycler® 480 SYBR Green I Master (2X Mix)	5 x 1 ml	04 707 516 001
NimbleGen Sequence Capture Hybridization Kit	■ 2X SC Hybridization Buffer ■ SC Hybridization Component A	05 340 721 001
NimbleGen Sequence Capture Wash and Elution Kit	■ 2X Stringent Wash Buffer ■ 10X SC Wash Buffer I ■ 10X SC Wash Buffer II ■ 10X SC Wash Buffer III ■ Elution Reagent*	05 340 730 001
PCR grade water	4 x 25 ml	03 315 843 001

* Reagent will not be used in the protocol described in this *User's Guide*.

Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
Illumina Paired-End Genomic DNA Sample Prep Kit	Illumina	1 kit	PE-102-1001
Agilent DNA 1000 Kit	Agilent	1 kit	5067-1504

Component	Supplier	Package Size	Catalog No.
Phusion High-Fidelity PCR Master Mix with HF Buffer	NEB (Finnzymes)	100 reactions -or- 500 reactions	F-531S -or- F-531L
QIAquick PCR Purification Kit	Qiagen	250	28106
1M Trizma hydrochloride (pH 7.8)	Sigma	1 liter	T2913-1L
5M Sodium chloride solution	Sigma	250 ml	71386
0.5M Ethylenediaminetetraacetic acid disodium salt solution (EDTA)	Sigma	100 ml	E7889-100ML
Dynabeads M-270 Streptavidin	Invitrogen	2 ml -or- 10 ml	653-05 -or- 653-06
Tubes: ■ 0.2 ml PCR tubes ■ 1.5 ml microcentrifuge tubes	*Multiple Vendors*		

Custom Oligonucleotides Purchased from IDT or Another Vendor

Component	Concentration	Sequence	Note(s)
PE-PRE1 Oligo[1, 2]	100 µM	5' - AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T - 3'	Asterisk (*) notation corresponds to addition of phosphorothioate bond before addition of the last "T" as a preventative measure against degradation in storage. This notation is consistent with IDT's submission format for synthesis. These oligos must be purified by HPLC following synthesis.
PE-PRE2 Oligo[1, 2]	100 µM	5' - CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC* T - 3'	
PE-HE1 Oligo[1, 2]	1000 µM	5' - AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T - 3'	HE PE1 and PE2 oligos must be resuspended in PCR grade water. Asterisk (*) notation corresponds to addition of phosphorothioate bond before addition of the last "T" as a preventative measure against degradation in storage. This notation is consistent with IDT's submission format for synthesis. These oligos must be purified by HPLC following synthesis.
PE-HE2 Oligo[1, 2]	1000 µM	5' - CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC* T - 3'	
PE-POST1 Oligo[1, 2]	100 µM	5' - AAT GAT ACG GCG ACC ACC GAG A -3'	
PE-POST2 Oligo[1, 2]	100 µM	5' - CAA GCA GAA GAC GGC ATA CGA G - 3'	

Component	Concentration	Sequence	Note(s)
qPCR NSC-0237, forward, Oligo	2 µM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'	These oligos (primers) are used in qPCR analysis, described in *Chapter 8*.
qPCR NSC-0237, reverse, Oligo	2 µM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'	
qPCR NSC-0247, forward, Oligo	2 µM	5' - CCC ACC GCC TTC GAC AT - 3'	
qPCR NSC-0247, reverse, Oligo	2 µM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'	
qPCR NSC-0268, forward, Oligo	2 µM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'	
qPCR NSC-0268, reverse, Oligo	2 µM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'	
qPCR NSC-0272, forward, Oligo	2 µM	5' - CAG CCC CAG CTC AGG TAC AG - 3'	
qPCR NSC-0272, reverse, Oligo	2 µM	5' – ATG ATG CGA GTG CTG ATG ATG - 3'	

1 Oligonucleotide sequences are the copyright of Illumina, Inc. (© 2006-2008). All rights reserved.

2 PE = Paired End; HE = Hybridization Enhancing; PRE = Pre-Capture; POST = Post-Capture

Oligonucleotide can be resuspended in PCR grade water or TE buffer unless otherwise noted.

Technical Support

For technical questions, contact your local Roche Microarray Technical Support. Refer to *www.nimblegen.com/arraysupport* for contact information.

Conventions Used in This Manual

Text Conventions

To impart information that is consistent and memorable, the following text conventions are used in this *User's Guide*:

Convention	Description
Numbered listing	Steps in a procedure that must be performed in the order listed.
Italic type, blue	Points to a different chapter in this *User's Guide* to consult or to a web site.

Symbols

The following types of notices may be used in this manual to highlight important information or to warn the operator of a potentially dangerous situation:

Symbol	Description
	Important Note. Used to bring your attention to important annotation.
	Information Note: Designates a note that provides additional information concerning the current topic or procedure.

Notes

Chapter 2. Storage of the SeqCap EZ Exome Library

Chapter 2 describes the aliquoting of and storage conditions for the SeqCap EZ Exome Library (referred to as "Exome Library" in the remainder of this *User's Guide*).

Upon receipt, undertake the following steps to ensure the highest performance of the Exome Library:

1. If frozen, thaw the Exome Library on ice.
2. Vortex the Exome Library for 3 seconds.
3. Centrifuge the tube of Exome Library at 10,000 x g for 30 seconds to ensure that the liquid is at the bottom of the tube before opening the tube.
4. Aliquot the Exome Library into single-use aliquots (4.5 µl/aliquot) in 0.2 ml PCR tubes (or 96-well plates if following the higher throughput protocol described in *Appendix C*) and store at -15°C to -25°C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.

The Exome Library should not undergo multiple freeze/thaw cycles. To help ensure the highest performance of the Exome Library, Roche NimbleGen recommends aliquoting the Exome Library into single-use volumes to prevent damage from successive freeze/thaw cycles.

Notes

Chapter 3. Sample Library Preparation

Chapter 3 describes the sample library preparation method and how to assess the quality of the sample library before amplification using LM-PCR.

Sample library preparation using the Illumina Paired-End Genomic DNA Sample Prep Kit requires 1 - 5 µg of sample library.

References

- Illumina Paired-End Genomic DNA Sample Prep Kit protocol ("Preparing Samples for Paired-End Sequencing," September 2009 or later)
- Agilent DNA 1000 Kit Guide

Step 1. Sample Library Preparation

1. Construct the sample library following the procedure described in the Illumina "Paired-End Sequencing Sample Preparation Guide" protocol. Follow the protocol from "Fragment DNA" through "Purify Ligation Products" with the following exceptions:
   - For a higher throughput alternative to nebulization, *Appendix A* of this *User's Guide* provides an optimized procedure for sonication that may also be used to generate DNA fragments for library construction.
   - Trim the gel as much as possible so that the gel slice weighs no more than 400 mg.
   - During the gel purification step, use the Qiagen Gel Extraction Kit as recommended in the protocol except incubate the gel piece in Qiagen buffer QG at room temperature for 5 - 10 minutes instead of incubating at 50°C. (Incubation at 50°C could result in a GC-bias in the downstream sequencing data. Refer to Quail et al., *Nature Methods,* 2008 Dec; 5(12):1005-10.)
   - Elute the DNA in 30 µl of Qiagen buffer EB as recommended.

     

     1 µl of the eluate is used for sample library quality assessment in Step 2 below. The remainder of the eluate is the sample library DNA to use as template in *Chapter 4. Sample Library Amplification using LM-PCR*.

Step 2. Sample Library Quality Assessment

1. Use 1 µl of the eluate from Step 1 above as input into the "Enrich DNA Fragments" protocol (page 25) in the Illumina "Paired-End Sequencing Sample Preparation Guide."

   

   The "Enrich DNA Fragments" protocol in the Illumina "Paired-End Sequencing Sample Preparation Guide" is used here for Sample Library quality assessment purposes only. The resulting amplified material is not used for any other purposes in this *User's Guide.*

2. Following Step 6 of the "Enrich DNA Fragments" protocol, purify the DNA using a Qiagen MinElute Purification Kit. Elute the DNA using 10 µl of buffer EB. Proceed with step 3 below.

3. Analyze the purified, amplified DNA library by running 1 µl on an Agilent DNA 1000 chip (Figure 2). The DNA library should have a peak between 200 - 400 bp.

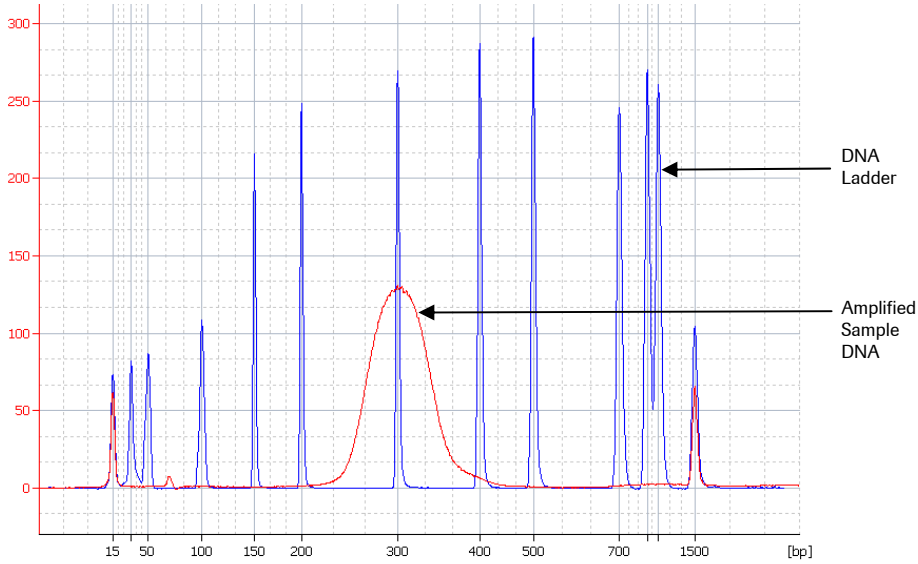

Figure 2: Example of an Amplified Paired-End Library DNA Sample Run on an Agilent DNA 1000 Chip

4. If the library passes this quality assessment step, proceed to *Chapter 4. Sample Library Amplification Using LM-PCR*.

Chapter 4. Sample Library Amplification Using LM-PCR

This chapter describes how to amplify the sample library (prepared in *Chapter 3*) using LM-PCR in preparation for hybridization to the Exome Library.

References

- Phusion High-Fidelity PCR Master Mix with HF Buffer Kit protocol (New England Biolabs - Finnzymes)
- Thermocycler Manual
- QIAquick Spin Handbook (Qiagen)
- Agilent DNA 1000 Kit Guide

Sample Requirements

For each sample library to be captured, the entire sample library from *Chapter 3*, Step 1, (approximately 30 µl total) is amplified via Pre-Capture LM-PCR.

Step 1. Prepare the LM-PCR

The Pre-Capture LM-PCR Master Mix is temperature sensitive. Thawing of components and preparation of LM-PCR reactions must be performed on ice.

1. Prepare the LM-PCR Master Mix in a 1.5 ml microcentrifuge (or 15 ml conical) tube. The amount of each reagent needed is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance):

Pre-Capture LM-PCR Master Mix	Per Each Individual Sample Library or Negative Control	48 Sample Libraries
Phusion High-Fidelity PCR Master Mix (2x)	50 µl	2,400 µl
PCR grade water	16 µl	768 µl
PE-PRE1 Oligo, 100 µM (Final Conc.: 2 µM)	2 µl	96 µl
PE-PRE2 Oligo, 100 µM (Final Conc.: 2 µM)	2 µl	96 µl
Total	**70 µl**	**3,360 µl**

2. Pipette 70 µl of LM-PCR Master Mix into each PCR tube or well.

3. Add the 30 µl of sample library (or PCR grade water for negative control) to the PCR tube or each well of the 96-well plate containing the LM-PCR Master Mix. (If less than 30 µl of sample library is available, add PCR grade water to make up the difference.) Mix well by pipetting up and down 5 times.

"Sample library" refers to the DNA from the "Purify Ligation Products" step, not the DNA from the "Enrich DNA Fragments" step, of the Illumina "Paired-End Sequencing Sample Preparation Guide." Refer to *Chapter 3. Sample Library Preparation* for more details.

Step 2. Perform PCR Amplification

1. Place the PCR tube (or 96-well PCR plate) in the thermocycler.
2. Amplify the sample library using the following Pre-Capture LM-PCR program:
   - Step 1: 30 seconds @ 98°C
   - Step 2: 10 seconds @ 98°C
   - Step 3: 30 seconds @ 65°C
   - Step 4: 30 seconds @ 72°C
   - Step 5: Go to Step 2, repeat 7 times
   - Step 6: 5 minutes @ 72°C
   - Step 7: Hold @ 4°C
3. Store the reaction at 4°C until ready for cleanup, up to 72 hours.

Step 3. Clean up the Amplified Sample Library

1. Transfer each amplified sample library into one 1.5 ml microcentrifuge tube (approximately 100 µl). Process the negative control in exactly the same way as the amplified sample library.
2. Follow the instructions provided with the Qiagen QIAquick PCR Purification Kit with the following modifications (listed below in Steps 3.3 - 3.8).
3. To each tube add 500 µl (5x) of Qiagen buffer PBI. Mix well by pipetting up and down 10 times.
4. Pipette the 600 µl of the amplified sample library in PBI into a QIAquick PCR Purification column.
5. Centrifuge at 10,000 x g for 30 - 60 seconds. Discard the flow-through.
6. Add 750 µl of PE buffer to the column. Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
7. Place the QIAquick column back in the same tube. Centrifuge the column for an additional minute.
8. Add 50 µl of PCR grade water directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube. Centrifuge at 10,000 x g for 1 minute to elute the DNA.

It is critical that the amplified sample library be eluted with PCR grade water and not buffer EB or 1X TE.

Step 4. Check the Quality of the Amplified Sample Library

1. Measure the A_{260}/A_{280} ratio of the amplified sample library to quantify the DNA concentration using a NanoDrop spectrophotometer and determine the DNA quality.

   - The A_{260}/A_{280} ratio should be 1.7 - 2.0.
   - The sample library yield should be ≥ 1.0 µg.
   - The negative control yield should be negligible.

2. Run 1 µl of each amplified sample library (and any negative controls) on an Agilent DNA 1000 chip. Run the chip according to manufacturer's instructions.

   The Bioanalyzer should indicate that most of the fragments fall between 200 - 400 bp (Figure 3). The negative control should not show any significant signal, which could indicate contamination between amplified sample libraries.

3. If the amplified sample library meets these requirements, proceed to *Chapter 5. Hybridization of Sample and Exome Libraries*. If the amplified sample library does not meet these requirements, reconstruct the library.

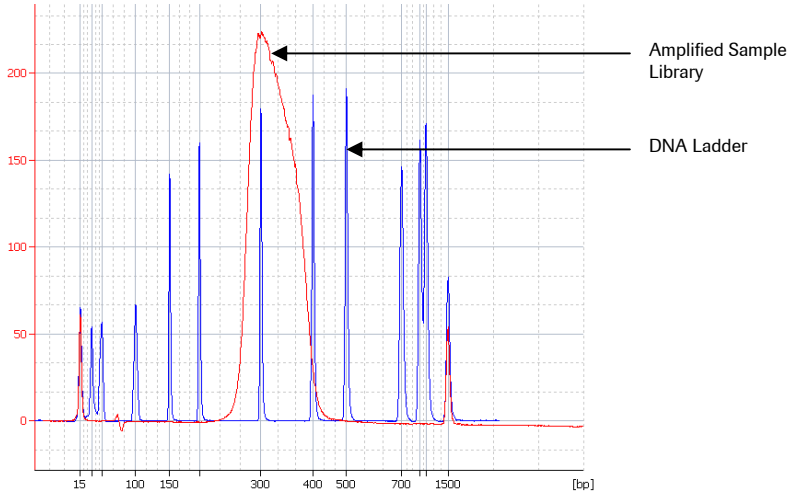

Figure 3: Example of an Amplified Sample Library Analyzed Using an Agilent DNA 1000 Chip

If there are any concerns at this stage regarding the quality of the amplified sample library, proceed to *Appendix B. Using qPCR for Amplified Sample Library Quality Control*.

Notes

Chapter 5. Hybridization of Sample and Exome Libraries

Chapter 5 describes the Roche NimbleGen protocol for hybridization of the amplified sample library and the Exome Library. Be aware of the following:

The hybridization protocol requires a thermocycler capable of maintaining 47°C for 64 - 72 hours.

Step 1. Prepare for Hybridization

1. Turn on a heat block to 95°C and let it equilibrate to the set temperature.
2. Remove the appropriate number of 4.5 µl Exome Library aliquots (1 per sample library) from the -15°C to -25°C freezer and allow them to thaw on ice.

Step 2. Prepare the Hybridization Sample

1. Add 5 µl of 1 mg/ml COT DNA and 1 µg of amplified sample library to a new 1.5 ml tube.
2. Add 1 µl of each 1,000 µM PE-HE1 and PE-HE2 Oligos to the amplified sample library plus COT DNA. (PE = Paired End; HE = Hybridization Enhancing).
3. Close the tube's lid and make a hole in the top of the tube's cap with an 18 - 20 gauge or smaller needle.

   

   The closed lid with a hole in the top of the tube's cap is a precaution to suppress contamination in the DNA vacuum concentrator.
4. Dry the amplified sample library/COT DNA/PE-HE Oligos in a DNA vacuum concentrator on high heat (60°C).

   

   Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.
5. To each dried-down amplified sample library/COT DNA/PE-HE Oligos, add:

   - 7.5 µl of 2X SC Hybridization Buffer
   - 3 µl of SC Hybridization Component A

   The tube with the amplified sample library/COT DNA/PE-HE Oligos should now contain the following components:

Component	Solution Capture
COT DNA	5 µg
Amplified sample library	1 µg
1,000 µM PE-HE1 and PE-HE2 Oligos	1,000 pmol each
2X SC Hybridization Buffer	7.5 µl
SC Hybridization Component A	3 µl
Total	**10.5 µl**

6. Cover the hole in the tube's cap with a sticker or small piece of laboratory tape.
7. Vortex the amplified sample library/COT DNA/PE-HE Oligos plus Hybridization Cocktail (2X SC Hybridization Buffer + SC Hybridization Component A) for 10 seconds and centrifuge at maximum speed for 10 seconds.
8. Place the amplified sample library/COT DNA/PE-HE Oligos/Hybridization Cocktail in a 95°C heat block for 10 minutes to denature the DNA.
9. Centrifuge the amplified sample library/COT DNA/PE-HE/Hybridization Cocktail at maximum speed for 10 seconds at room temperature.
10. Transfer the amplified sample library/COT DNA/PE-HE Oligos/Hybridization Cocktail to the 4.5 µl aliquot of Exome Library in a 0.2 ml PCR tube prepared in *Chapter 2* (entire volume can also be transferred to a 96-well PCR plate).
11. Vortex for 3 seconds and centrifuge at maximum speed for 10 seconds.

    The hybridization sample should now contain the following components:

Component	Solution Capture
COT DNA	5 µg
Amplified Sample Library	1 µg
1,000 µM PE-HE1 and PE-HE2 Oligos	1,000 pmol each
2X SC Hybridization Buffer	7.5 µl
SC Hybridization Component A	3 µl
Exome Library	4.5 µl
Total	**15 µl**

12. Incubate in a thermocycler at 47°C for 64 - 72 hours. The thermocycler's heated lid should be turned on and set to maintain 57°C (+10°C above the hybridization temperature).

Chapter 6. Washing and Recovery of Captured DNA

Chapter 6 describes the process for the washing and recovery of the captured DNA from the hybridization of the amplified sample library and Exome Library. (Refer to *Appendix C* for instructions for increased throughput applications.)

It is extremely important that the water bath temperature be closely monitored and remains at 47°C. Because the displayed temperatures on many water baths are often imprecise, Roche NimbleGen recommends that you place an external, calibrated thermometer in the water bath.

Equilibrate buffers at 47°C for at least 2 hours before washing the captured DNA.

Step 1. Prepare Sequence Capture Wash Buffers

1. Dilute 10X SC Wash Buffers (I, II, and III) and 2X Stringent Wash Buffer to create 1X working solutions.

Amount of Concentrated Buffer	Amount of PCR Grade Water	Total Volume of 1X Buffer*
10 ml - 2X Stringent Wash Buffer	10 ml	**20 ml**
2 ml - 10X SC Wash Buffer I	18 ml	**20 ml**
1 ml - 10X SC Wash Buffer II	9 ml	**10 ml**
1 ml - 10X SC Wash Buffer III	9 ml	**10 ml**

* Store working solutions at room temperature for up to 2 weeks.

2. Preheat the following wash buffers:
   - 20 ml of Stringent Wash Buffer heated to 47°C in a water bath.
   - 5 ml of SC Wash Buffer I heated to 47°C in a water bath.

Step 2. Prepare Streptavidin Dynabead Binding and Wash Buffer

1. Prepare the Streptavidin Dynabead Binding and Wash Buffer in either a 15 ml or 50 ml conical tube:

Component	4 Captures*	48 Captures*
1 M Trizma hydrochloride (pH 7.8)	25 µl	245 µl
0.5 M EDTA	5 µl	49 µl
5 M NaCl	1,000 µl	9,800 µl
PCR grade water	1,470 µl	14,406 µl
Total	**2.5 ml**	**24.5 ml**

* Volume adjusted for pipetting variance. Store wash buffer at room temperature for up to 2 months.

2. Vortex for 20 seconds and label the tube appropriately.
3. Store the Streptavidin Dynabead Binding and Wash Buffer at room temperature while proceeding immediately to "Step 3, Prepare the Streptavidin Dynabeads."

Step 3. Prepare the Streptavidin Dynabeads

1. Allow the Streptavidin Dynabeads to warm to room temperature for 30 minutes prior to use.
2. Mix the beads thoroughly by vortexing for 1 minute.
3. Aliquot 100 µl of beads for each capture into a single 1.5 ml tube (i.e. for 1 capture use 100 µl beads and for 4 captures use 400 µl beads, etc.). Enough beads for 6 captures can be prepared in a single tube.
4. Place the tube in a DynaMag-2 device. When the liquid becomes clear (should take less than 5 minutes), remove and discard the liquid being careful to leave all of the beads in the tube. Any remaining traces of liquid will be removed with subsequent wash steps.
5. While the tube is in the DynaMag-2 device, add twice the initial volume of beads of Streptavidin Dynabead Binding and Wash Buffer (i.e. for 1 capture use 200 µl of buffer and for 4 captures use 800 µl buffer, etc.).
6. Remove the tube from the DynaMag-2 device and vortex for 10 seconds.
7. Place the tube back in the DynaMag-2 device to bind the beads. Once clear, remove and discard the liquid.
8. Repeat Steps 3.5 - 3.7 for a total of 2 washes.
9. After removing the buffer following the second wash, resuspend by vortexing the beads in 1x the original volume using the Streptavidin Dynabead Binding and Wash Buffer (i.e. for 1 capture use 100 µl buffer and for 4 captures use 400 µl buffer, etc.) that was prepared in Step 2.3.
10. Aliquot 100 µl of resuspended beads into new 0.2 ml tubes.
11. Use the DynaMag-2 device to bind the beads by holding the tube against the magnet. Remove and discard the liquid when clear.
12. The Streptavidin Dynabeads are now ready to bind the captured DNA. Proceed immediately to "Step 4, Bind DNA to the Streptavidin Dynabeads."

After Step 3.11, it is very important to proceed to Step 4 as quickly as possible. Do not allow the Streptavidin Dynabeads to dry out. Small amounts of residual Streptavidin Dynabead Binding and Wash Buffer will not interfere with binding of DNA to the Streptavidin Dynabeads.

Step 4. Bind DNA to the Streptavidin Dynabeads

1. Transfer the hybridization samples to the Streptavidin Dynabeads prepared in Step 3.12 of this chapter.
2. Mix thoroughly by pipetting up and down 10 times.
3. Bind the captured sample to the beads by placing the tubes containing the beads and DNA in a thermocycler set to 47°C for 45 minutes (heated lid set to 57°C). Mix the samples by vortexing for 3 seconds at 15 minute intervals to ensure that the beads remain in suspension. It is helpful to have a vortex mixer located close to the thermocycler for this step.

Step 5. Wash the Streptavidin Dynabeads Plus Bound DNA

1. After the 45-minute incubation, transfer the entire content of each 0.2 ml tube to a 1.5 ml tube.
2. Use the magnet from the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
3. Add 100 µl of SC Wash Buffer I heated to 47°C.
4. Mix by vortexing for 10 seconds.
5. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
6. Remove the tubes from the DynaMag-2 device and add 200 µl of Stringent Wash Buffer heated to 47°C. Pipette up and down 10 times to mix. Work quickly so that the temperature does not drop much below 47°C.
7. Incubate at 47°C for 5 minutes.
8. Repeat Steps 5.5 - 5.7 for a total of 2 washes with Stringent Wash Buffer heated to 47°C.
9. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
10. Add 200 µl of room temperature SC Wash Buffer I and mix by vortexing for 2 minutes. If liquid has collected in the tube's cap, tap the tube gently to collect the liquid into the tube's bottom before continuing to the next step.
11. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
12. Add 200 µl of room temperature SC Wash Buffer II and mix by vortexing for 1 minute.
13. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
14. Add 200 µl of room temperature SC Wash Buffer III and mix by vortexing for 30 seconds.

15. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.

16. Remove the tubes from the DynaMag-2 device and add 50 µl PCR grade water to each tube of bead-bound captured sample.

17. Store the beads plus captured samples at -15°C to -25°C or proceed to *Chapter 7. Captured DNA Amplification Using LM-PCR*.

There is no need to elute DNA off the beads. The beads plus captured DNA will be used as template in the LM-PCR as described in *Chapter 7*.

Chapter 7. Captured DNA Amplification Using LM-PCR

Chapter 7 describes the amplification of captured DNA, bound to the Streptavidin Dynabeads, using LM-PCR. A total of 2 reactions are performed per sample, and subsequently combined, to minimize PCR bias.

References

- Phusion High-Fidelity PCR Master Mix with HF Buffer Kit protocol (New England Biolabs - Finnzymes)
- Thermocycler Manual
- QIAquick Spin Handbook (Qiagen)
- Agilent DNA 1000 Kit Guide

Step 1. Prepare the LM-PCR

The Post-Capture LM-PCR Master Mix and the individual PCR tubes must be prepared on ice.

1. Prepare the Post-Capture LM-PCR Master Mix in a 1.5 ml tube. The amount of each reagent needed for 2 reactions (1 captured DNA sample) is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance):

Post-Capture LM-PCR Master Mix	2 Reactions (for 1 captured DNA sample or negative control)
Phusion High-Fidelity PCR Master Mix (2x)	100 µl
PCR grade water	52 µl
PE-POST1 Oligo, 100 µM (Final Conc.: 2 µM)	4 µl
PE-POST2 Oligo, 100 µM (Final Conc.: 2 µM)	4 µl
Total	**160 µl**

2. Pipette 80 µl of Post-Capture LM-PCR Master Mix into the reaction tubes or wells.
3. Vortex the bead-bound captured DNA to ensure a homogenous mixture of beads.
4. Aliquot 20 µl of bead-bound captured DNA as template into each of the 2 PCR tubes/wells. Add 20 µl of PCR grade water to the negative control. Mix well by pipetting up and down 5 times.

Step 2. Perform PCR Amplification

1. Place PCR tubes/plate in the thermocycler.
2. Amplify the captured DNA using the following Post-Capture LM-PCR program:
   - Step 1: 30 seconds @ 98°C
   - Step 2: 10 seconds @ 98°C
   - Step 3: 30 seconds @ 60°C
   - Step 4: 30 seconds @ 72°C
   - Step 5: Go to Step 2, repeat 17 times
   - Step 6: 5 minutes @ 72°C
   - Step 7: Hold @ 4°C
3. Store reactions at 4°C until ready for purification, up to 72 hours.

Step 3. Clean up the Amplified Captured DNA

1. Pool the two reactions from each amplified captured DNA sample into a single 1.5 ml microcentrifuge tube (approximately 200 µl). Process the negative control in exactly the same way as the amplified captured DNA.
2. Follow the instructions provided with Qiagen QIAquick PCR Purification Kit with the following modifications (listed below in Steps 3.3 - 3.9).
3. To each tube, add 1,000 µl (5x volume) of Qiagen buffer PBI. Mix well.
4. Pipette 750 µl of the amplified captured DNA in PBI into a QIAquick PCR Purification column.
5. Centrifuge at ≥ 10,000 x g for 30 - 60 seconds. Discard the flow-through.
6. Load the remainder of the amplified captured DNA in PBI into the same column and centrifuge at ≥ 10,000 x g for 30 - 60 seconds. Discard the flow-through.
7. Add 750 µl of buffer PE to the column. Centrifuge at ≥ 10,000 x g for 1 minute.
8. Discard the flow-through and place the column back in the same tube. Centrifuge the column for an additional minute.
9. Add 50 µl of buffer EB directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube. Let the column stand for 1 minute. Centrifuge at ≥ 10,000 x g for 1 minute to elute the DNA.

Step 4. Determine the Concentration, Size Distribution, and Quality of the Amplified Captured DNA

1. Analyze 1 µl of the amplified captured DNA and negative control using an Agilent DNA 1000 chip and measure the A_{260}/A_{280} ratio using a NanoDrop spectrophotometer to quantify the concentration of DNA and to determine the DNA quality. The negative control should not show significant amplification, which could be indicative of contamination. Amplified captured DNA should exhibit the following characteristics:

   - A_{260}/A_{280}: 1.7 - 2.0
   - LM-PCR yield > 1.0 µg
   - Average fragment length between 200 - 400 bp

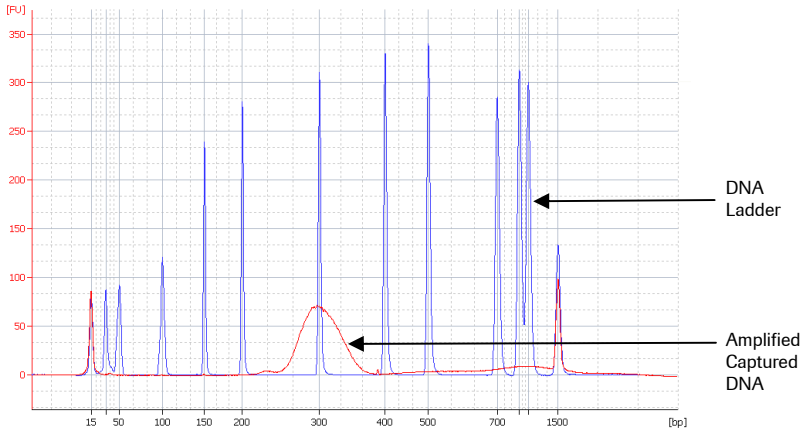

Figure 4: Example of Successfully Amplified Captured DNA Analyzed Using an Agilent DNA 1000 Chip

2. If the amplified captured DNA meets the requirements, proceed to *Chapter 8. Measurement of Enrichment Using qPCR*.

   If the amplified captured DNA does not meet the A_{260}/A_{280} ratio requirement, purify again using a second Qiagen QIAquick PCR Purification column.

3. If the results of the qPCR assays in *Chapter 8* indicate the enrichment was successful, the amplified captured DNA is ready for sequencing.

Notes

Chapter 8. Measurement of Enrichment Using qPCR

Chapter 8 describes the qPCR assays used to estimate relative fold-enrichment by measuring the relative abundance of control targets in amplified sample library and amplified captured DNA. These assays are an inexpensive way to determine whether the capture was successful.

A standardized set of qPCR SYBR Green assays are employed as internal quality controls for NimbleGen Sequence Capture experiments performed with human total gDNA. The genomic loci recognized by these assays are included as capture targets in every Exome Library. Comparison by qPCR of the relative DNA concentrations of these control loci in the amplified sample library and amplified captured DNA allows for the estimation of enrichment of a capture target before committing to expensive and/or time-consuming downstream applications.

The internal control region assays recommended in this chapter were selected due to their convenience for use across different species and because they produce consistent results. It is worth considering designing and evaluating locus-specific qPCR assays for your own region(s) of interest because capture results can vary from locus to locus. It is possible that your regions of interest may enrich differently than our internal control regions.

For more information regarding general PCR and qPCR methods, consult the PCR Applications Manual (3rd Edition), available from the Roche Applied Science website (*www.roche-applied-science.com*).

Step 1. Perform Advance Preparations

1. Determine the number of DNA samples to be analyzed.

A "DNA sample" in this chapter is defined as one Amplified Sample Library (*Chapter 4*) and the corresponding Amplified Captured DNA (*Chapter 7*). Assuming a standard set of 4 NimbleGen Sequence Capture (NSC) control locus qPCR assays will be used and qPCR assays will be performed in triplicate, each DNA Sample will require 24 individual qPCR reactions for analysis. One negative control (i.e. no-template-control, NTC) should always be included to monitor for contamination in qPCR assay primers, other qPCR reagents, and the entire PCR process. One positive control template, ideally consisting of the original genomic DNA starting material, should always be included to verify assay function. The negative and positive controls will each require 12 additional qPCR reactions (refer to Figure 5).

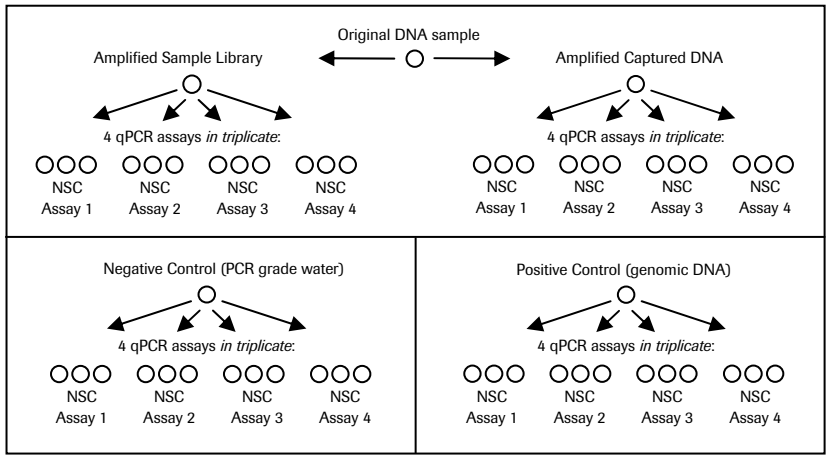

Figure 5: qPCR Experimental Overview

2. The number of reaction wells required to perform a complete control locus qPCR analysis is determined by the formula, **W = 24 x (S + 1)**, where **W** is the total number of wells required and **S** is the number of original DNA samples in the experiment (1 per capture). For example, analyzing 1 captured DNA sample, 1 negative control, and 1 positive control with 4 NSC assays performed in triplicate requires 48 individual qPCR reactions, **W = 24 x (1 + 1)**. Analyzing 2 captured DNA samples requires 72 qPCR reactions, **W = 24 x (2 + 1)**, etc.
3. Dilute the NSC assay forward and reverse primers to 2 µM.
4. Dilute sufficient amounts of amplified sample library and amplified captured DNA to a concentration of 5 ng/µl in PCR grade water for use as qPCR templates (approximately 100 µl each).
5. Add the following for a 15 µl NSC assay (for larger volume reactions, adjust the reagent amounts proportionally):
   - 5.9 µl of PCR grade water
   - 0.3 µl of NSC Assay forward primer (2 µM)
   - 0.3 µl of NSC Assay reverse primer (2 µM)
   - 7.5 µl of SYBR Green Master (2X)
   - 1 µl of 5 ng/µl template (amplified sample library, amplified captured DNA, positive control genomic DNA, or negative control PCR grade water, templates. The final concentration of templates in the reaction should be 0.333 ng/µl, except for the negative control PCR grade water template.)
6. Program the qPCR instrument using the conditions specified in Table 1.

   These conditions are optimized for use with the LightCycler® 480 Instrument II and LightCycler® 480 SYBR Green I 2X Master Mix. The use of a different thermocycler or reagents could require altering these conditions to achieve optimal results.

Table 1: qPCR Instrument Cycling Conditions

Program Name	Cycles	Analysis Mode	Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Acquisition mode
Pre-incubation	1	None	95	00:10:00	4.8	---	None
Amplification	40	Quantification	95	00:00:10	4.8	---	None
			60	00:01:00	2.5	---	Single
Melting Curve	1	Melting Curves	95	00:00:10	4.8	---	None
			65	00:01:00	2.5	---	None
			95	---	---	5	Continuous
Cooling	1	None	40	00:00:10	2	---	None

Table 2: Recommended NSC qPCR Assays. Forward and reverse primer sequences for 4 different NSC control locus assays are shown in Table 2. All of these assays have been confirmed on the SeqCap EZ Exome Library using human captured DNA.

NSC qPCR Assay Name	Primer Sequences (5′ → 3′)	T_m (°C)	Product Length	qPCR Efficiency (*E*)
NSC-0237	F: CGCATTCCTCATCCCAGTATG R: AAAGGACTTGGTGCAGAGTTCAG	81.15	80 bp	1.84
NSC-0247	F: CCCACCGCCTTCGACAT R: CCTGCTTACTGTGGGCTCTTG	81.03	74 bp	1.80
NSC-0268	F: CTCGCTTAACCAGACTCATCTACTGT R: ACTTGGCTCAGCTGTATGAAGGT	78.99	75 bp	1.78
NSC-0272	F: CAGCCCCAGCTCAGGTACAG R: ATGATGCGAGTGCTGATGATG	82.23	71 bp	1.93

Control Locus NSC qPCR assays enable you to measure the enrichment of a small set of standardized capture control loci that represent a range of known capture efficiencies. These assays act as a proxy for estimating the enrichment of larger populations of capture targets without a need for sequencing. If qPCR analysis using NSC assays indicates a successful capture of the control loci, it is likely that the experimental loci of interest were also successfully captured.

It is recommended that the 4 assays listed in Table 2 are the minimum number used for analysis of a SeqCap EZ Exome Library experiment to obtain an adequate representation of the diverse population of capture target loci. However, analysis using additional assays that are custom designed by you to measure enrichment of your specific targets of interest could provide additional useful information.

Step 2. Set up Control Locus qPCR Reactions

1. Using the information provided in *Chapter 8*, Steps 1.1 - 1.3, set up the required number of NSC assays with amplified sample library templates, amplified captured DNA templates, negative control templates (PCR grade water), and positive control templates (gDNA). Set up the reactions inside a PCR hood to minimize contamination.
2. Run the reactions in the thermocycler using the cycling conditions listed in Table 1.
3. Following raw data collection, run the Absolute Quantification Analysis Module within the LightCycler® 480 Software, Version 1.5, using the 2nd Derivative Maximum Method. It is not necessary to run a template standard curve for the analysis unless you want to measure the efficiency of the qPCR assays. The raw Cp values will be used to perform a simplified version of *relative* quantification comparing amplified sample library and amplified captured DNA.
4. Perform a melting curve (dissociation) analysis to verify that nonspecific amplification products, primer dimers, and other artifacts are not contributing to the Cp values for any samples.
5. Copy or export the Cp values to a spreadsheet program for further analysis.

Cp (crossing point) values reported by the LightCycler® 480 Instrument II and software are analogous to the C_t (crossing threshold) values reported by other instruments and represent the cycle at which fluorescence signal in a reaction well rises above background fluorescence signals in that well. The Cp value measured for a sample is dependent on the initial concentration of template DNA in the reaction. Lower Cp values correspond to higher initial template concentrations.

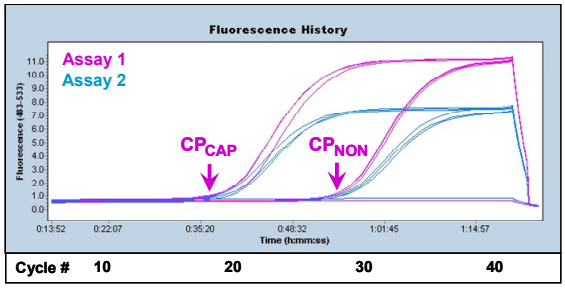

Figure 6: Example of Sequence Capture qPCR Data for Two NSC Assays Generated Using the LightCycler® 480 Instrument II. In a successful experiment, the Cp values from qPCR of amplified captured DNA templates (CAP) will be significantly lower than Cp values from amplified sample library templates (NON) for all assays.

Step 3. Analyze Data

1. Calculate the average Cp values for all replicate reactions.

Replicate Cp values obtained from qPCR of each different template should be similar. If the calculated standard deviation for 3 replicate Cp values is > 0.2 for a qPCR assay, you should consider repeating the assay to obtain more consistent results.

2. Confirm that the negative control reactions did not produce significant fluorescent signals, which might indicate a problem with PCR contamination resulting in difficulty interpreting experimental results.

   Primer dimer amplification can occasionally produce an increase in fluorescence signal in a negative control reaction in the absence of target template molecules, but this typically occurs very late in the run (i.e. Cp approaching 35 cycles), and it is easy to differentiate primer dimer from target amplicon by melting curve analysis (refer to Step 2.4). Primer dimers are typically indicated by a peak melting temperature significantly lower than the peak melting temperature of the correct amplicon. Primer dimers in a negative control reaction are not usually a concern because they are an artifact of low-affinity annealing in the absence of competing template. However, fluorescence created from the presence of primer dimer (as indicated by a second, lower T_m peak in the melting curve) in an experimental sample reaction can lead to an erroneously high estimate of template concentration in that sample, and sample(s) with multiple melting curve peaks should be regarded as suspect and omitted from Cp value determinations.

3. For each different sample and NSC assay combination, subtract the average Cp value measured for the amplified captured DNA template from the average Cp value measured for the corresponding amplified sample library template. This value is the delta-Cp. A successfully enriched amplified captured DNA sample should generate a lower Cp value then its corresponding amplified sample library. Thus the delta-Cp calculated from an NSC assay should be positive if the capture process enriched the corresponding locus.

4. Calculate the fold-enrichment for a NSC control locus by raising the PCR Efficiency (*E*) for that assay to the power of the delta-Cp measured for the corresponding control locus, or $E^{delta\text{-}Cp}$.

   When PCR assays operate at 100% theoretical efficiency (i.e. a perfect doubling of target sequences in every cycle), E = 2. The E values for the NSC assays listed in Table 2 were measured by Roche NimbleGen. However, because multiple parameters (PCR instrument, reagent lots, etc.) can affect the efficiency of a PCR assay, it is recommended to determine E values empirically in your own laboratory for each different NSC assay. The efficiency of NSC assays is determined in the same way as other PCR assays by applying linear regression analysis to amplification data from a template standard dilution series. The slope of the standard curve is used to determine E with the equation $E = 10^{(-1/slope)}$. A slope of -3.3 indicates an E value of 2. Calculated E values less than 2 are common. The software provided with most qPCR instruments can perform calculations of E automatically, or they may be calculated using external mathematical or spreadsheet software.

Example: NSC-0268 assay (assuming *E* = 1.78)

- Replicate Cp values for qPCR of amplified sample library = 28.3, 28.5, 28.4
- Replicate Cp values for qPCR of amplified captured DNA = 17.5, 17.3, 17.7
  - Average $Cp_{amplified\ sample\ library}$ = 28.4
  - Average $Cp_{amplified\ captured\ DNA}$ = 17.5
  - Delta-Cp = 10.9
  - Fold enrichment ($E^{delta\text{-}Cp}$) = $(1.78)^{10.9}$ = **537**

Example: NSC-0272 assay (assuming $E = 1.93$)

- Replicate Cp values for qPCR of amplified sample library = 28.0, 28.2, 28.1
- Replicate Cp values for qPCR of amplified captured DNA = 18.2, 18.1, 18.0
  - Average $Cp_{\text{amplified sample library}} = 28.1$
  - Average $Cp_{\text{amplified captured DNA}} = 18.1$
  - Delta-Cp = 10
  - Fold enrichment $(E^{\text{delta-Cp}}) = (1.93)^{10} = 717$

Average Cp values for negative control (PCR grade water) assays should be negligible, or they may indicate the presence of cross-contamination among wells or reagent contamination. If this is observed, the qPCR experiment should be repeated.

Average Cp values for Positive Control (genomic DNA) assays should be similar (within approximately 1 Cp) to the average Cp values obtained for the qPCR of amplified sample library.

Interpreting qPCR Results

The theoretical maximum average fold-enrichment of a target the size of the human exome (~30 Mb) is approximately 100-fold. However, probes targeting the NSC control loci were designed to facilitate enrichments greater than 100-fold, and so their reported qPCR values should not be interpreted as a literal estimate of the expected enrichment of other targeted loci. Rather, the NSC control assays are primarily intended as a screen for potentially poor enrichment results so that unproductive sequencing may be avoided. Using NSC control locus assay data from a series of SeqCap EZ Exome experiments, we calculated fold enrichment values for each of the four control assays and for the average of all four assays (Table 3). These data were compared to the sequence data for the experiments. Based on the results, we recommend not sequencing the captured DNA from any SeqCap EZ Exome experiment with one or more calculated fold enrichment values below those shown in Table 3. We recommend such experiments be repeated from the beginning.

The utility of this metric for identifying poor enrichment results is strongly dependent on the use of good qPCR technique, correct qPCR efficiency (E) values and inclusion of appropriate experimental controls. A SeqCap EZ Exome experiment producing control locus fold enrichment values lower than those shown in Table 3 is not a guarantee of a failed enrichment, since genomic polymorphisms and other variables may affect qPCR amplification. Similarly, a SeqCap EZ Exome experiment producing control locus fold enrichment values greater than those shown in Table 3 is not a guarantee of a successful result.

Table 3: Recommended Metrics for Proceeding to Sequence Captured DNA from SeqCap EZ Exome Experiments. Based on experiments performed by Roche NimbleGen, we recommend that captured DNA from SeqCap EZ Exome experiments should not be sequenced if one or more calculated NSC control locus fold enrichment values, or the average of all four, are below the levels indicated in the table.

NSC Assay	NSC-237	NSC-247	NSC-268	NSC-272	Average
Fold enrichment cutoff value	250	50	300	300	400

Data Analysis Considerations

Careful consideration and data interpretation are necessary when deciding whether to sequence captured DNA. Consider the types of targets and how the control loci represent them, the region targeted for enrichment, and qPCR as an estimation platform.

The large difference between the fold enrichment values for the two example NSC assays (above) demonstrates the importance of testing multiple NSC control loci to obtain values more likely to accurately represent the larger population of capture targets. This difference is primarily influenced by two parameters: 1) differences in capture efficiency of the different loci, and 2) amplification efficiency differences between the two qPCR assays. Without determination of the efficiency of the qPCR assays in each run, the local differences in capture efficiency cannot be determined.

The efficiency of the qPCR assays can be variable across runs, which means that strict determination requires a standard dilution curve for each locus during each run. This *User's Guide* assumes that across a collection of loci, both the intrinsic locus capture efficiencies and the individual qPCR reaction efficiencies will settle in upon some common mean value. Therefore, employments of more loci yield better global estimates. It is not uncommon for highly efficient qPCR assays to demonstrate fold enrichment values on the order of 1,000 or more. However, benchmarking with such large values results in over-estimating global capture (i.e. pulls the average fold enrichment up too far). Also, platform error in qPCR measurements is ± 50% (or one cycle when comparing two measures) meaning that it takes many replicates to discriminate a 300-fold enrichment from a 600-fold enrichment. Consideration of this platform error means that 2-fold differences should be considered the same measure, rather than different measures of enrichment. Consider the size of your sequencing budget and establish qPCR benchmarks that fit your needs.

The average maximum enrichment level of capture targets within a large genome is dependent on the size of the capture target as a percentage of its genome. For example, if 50% of a genome were targeted for capture, an ideal result should yield no better than a 2-fold average enrichment for the targeted loci. Smaller capture targets in the same genome would have higher maximum average enrichment levels. Thus, the definition of a "successful" NimbleGen Sequence Capture experiment, as estimated by fold-enrichment values for control loci, might differ substantially with different sized capture targets or different downstream applications for the captured DNA.

Notes

Appendix A. Alternative Sonication Procedure for Illumina Paired-End Library Construction

This appendix describes a procedure for an increased throughput method of fragmenting genomic DNA for sample library preparation. This protocol was optimized using the Covaris E210 water bath sonicator and requires only 3 µg of genomic DNA as compared with the 5 µg suggested for Ilumina's nebulization procedure.

References

- Illumina Paired-End Genomic DNA Sample Prep Kit protocol ("Preparing Samples for Paired-End Sequencing," September 2009 or later)
- Covaris E210 Water Bath Sonicator User's Guide
- Agilent Bioanalyzer DNA 1000 Kit Guide
- QIAquick Spin Handbook (Qiagen)

Genomic DNA Fragmentation Using Sonication

1. Bring 3 µg of genomic DNA for each sample to a total volume of 100 µl with 1X TE buffer in a Covaris microTube.
2. Set up the Covaris Sono Lab sample prep system for the following sonication conditions:
    - Duty Cycle: 10%
    - Intensity: 5
    - Cycles per Burst: 200
    - Time: 180 s
    - Temp: 25°C
3. Load your samples in the Covaris Sono Lab sample prep system and set the software to recognize the samples.
4. Run the program to fragment the DNA.
5. After fragmentation, clean/concentrate each fragmented sample using a Qiagen QIAquick PCR Purification column (following the manufacturer's directions). Elute with 30 µl of buffer EB.
6. Load 1 µl per sample onto an Agilent DNA 1000 chip for confirmation of size distribution.
7. If DNA fragment distribution is satisfactory (median fragment size in the 100 - 500 bp range), continue with the Illumina paired-end library preparation beginning at the end repair step.

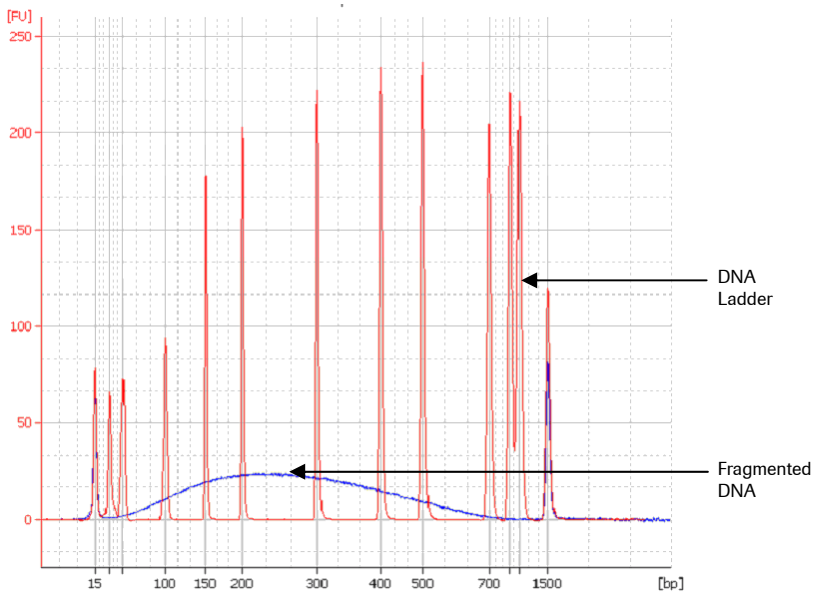

Figure 7: Example of DNA Size Distribution from Sample Fragmented by Covaris Bath Sonicator When Analyzed Using an Agilent DNA 1000 Chip

Appendix B. Using qPCR for Amplified Sample Library Quality Control

This appendix provides instructions for performing a qPCR-based procedure to measure the quality of the amplified sample library prior to hybridization. This quality control test is optional, and recommended for use particularly in these situations:

- The user is inexperienced with using the SeqCap EZ Exome Library SR protocol.
- The user is concerned about the quality of the input genomic DNA sample.
- The user is concerned about the quality of the amplified sample library following the Pre-Capture LM-PCR step.

This procedure is not suitable for identifying amplified sample libraries of only slightly reduced quality but in a limited number of cases has been able to identify significantly flawed amplified sample libraries

Step 1. Perform Advance Preparations & Set up qPCR Reactions

1. Using the Nanodrop concentration of the amplified sample library obtained in *Chapter 4*, Step 4.1, dilute a small portion of the amplified sample library to 5 ng/µl using PCR grade water (approximately 50 µl final volume).
2. Dilute a genomic DNA control (unamplified) to 5 ng/µl using PCR grade water (approximately 50 µl final volume).
3. Follow the instructions in *Chapter 8*, Step 1, to set up and perform Control Locus qPCR assays using as template: amplified sample library, genomic DNA (positive control), and PCR grade water (negative control).

Step 2. Data Analysis

The input material used for library construction is 100% genomic DNA. After fragmentation and ligation of adapters, the resulting library contains approximately 70% genomic DNA by mass, with the other 30% consisting of adapter DNA (the precise fraction depends on the distribution of DNA fragment sizes and the amount of adapter dimers present in the library preparation). Thus, assuming the same amount of DNA is used as template for qPCR, each amplification target in a genomic DNA template will have a slightly higher relative concentration than the same target in an LM-PCR amplified library template. Raw Cp values from qPCR analysis of various loci could reflect this difference, with LM-PCR amplified library templates generating slightly higher Cp values than control genomic DNA templates of the same mass. Furthermore, in a well-made genomic DNA library, all loci present in the starting genomic DNA will be present in the resulting library in the same proportion. Poor construction of a genomic DNA library, or poor LM-PCR amplification of a genomic DNA library, can alter these proportions significantly and result in allelic bias or even allele "drop out" in extreme cases.

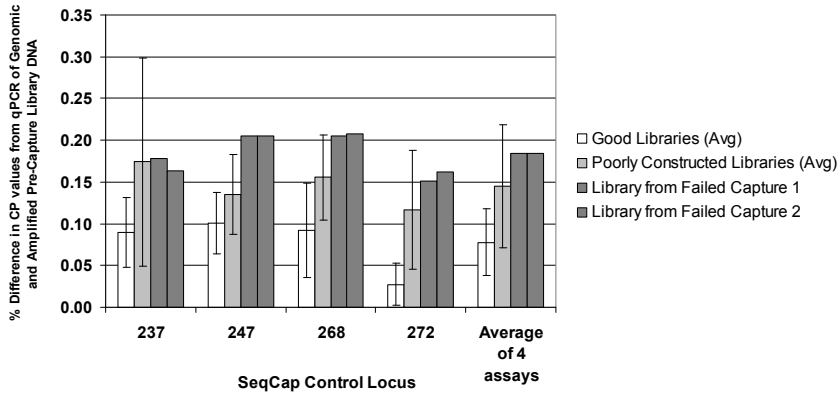

Figure 8: Use of qPCR to Check Amplified Library Quality Prior to Hybridization. Based on experiments performed by Roche NimbleGen, an LM-PCR amplified library may not be suitable for hybridization if the amplified library's Cp value is > 15% higher than the Cp value of the input genomic DNA in qPCR assays for two or more of the four standard control loci. The data was collected using the protocol and conditions described in *Chapter 8*. Error bars are ± 1 standard deviation. The % difference in Cp values from qPCR of genomic and amplified pre-capture library DNA is calculated using the formula: (Pre-Capture CP - Genomic CP) / Genomic CP

Figure 8 shows summarized results from qPCR analysis of four NSC control loci in several LM-PCR amplified libraries and their respective input genomic DNAs. In a series of capture experiments that were considered successful (i.e. "Good Library"), the average Cp values from qPCR of the amplified libraries were typically less than 10% higher than the Cp values from the input genomic DNAs. A series of libraries that were intentionally poorly constructed or amplified (e.g. low genomic DNA amounts used for library construction, low library amounts used as template for LM-PCR reactions, polymerase stored at improper temperature prior to LM-PCR, or combinations of these) yielded amplified library Cp values that were often greater than 10% higher than the Cp values from the corresponding genomic DNAs. Two libraries used in capture experiments that were ultimately considered failures showed a Cp value difference of greater than 15% for all four NSC control loci.

Based on these observations, Roche NimbleGen recommends that you do not hybridize the amplified library if qPCR using two or more NSC control assays yield Cp values from the amplified library that are greater than 15% higher than Cp values from the input genomic DNA. Instead, you should reconstruct and test the library again before proceeding. Application of these guidelines is expected to reduce but not completely eliminate the risk of poor sequence capture results related to library quality.

Appendix C. Higher Throughput, 96-Well Plate Processing

This appendix describes a procedure for an increased throughput method for Exome Library washing and recovery of captured DNA. This appendix can be substituted for *Chapter 6* of the protocol to process captured DNA in a 96-well plate format and to adapt the protocol onto a liquid handling instrument.

Additional Equipment, Labware & Consumables

Equipment	Supplier	Item Number
MagnaBot II Magnetic Separation Device	Promega	V8351
Multichannel Pipettors (20 µl and 200 µl)	*Multiple vendors*	
96-well PCR Plate (1/2 skirt)	*Multiple Vendors* *Note: Full skirt 96-well PCR plates will not work with the MagnaBot II Magnetic Separation Device.*	
15 ml and 50 ml Conical Tubes	*Multiple vendors*	

Step 1. Prepare Buffers

1. Prepare 50 ml of Bead Binding and Wash Buffer (enough for a full 96-well plate) in a 50 ml conical tube.

Component	Volume
1 M Trizma hydrochloride (pH 7.8)	500 µl
0.5 M EDTA	100 µl
5 M NaCl	20 ml
PCR grade water	29.4 ml
Total	**50 ml**

Store buffer at room temperature after prepared.

2. Prepare 50 ml of a 1X concentration buffer for each of the Sequence Capture Wash Buffers (enough of each buffer for a full 96-well plate) each in a separate 50 ml conical tube.

Component	Volume
2X Stringent Wash Buffer	25 ml
PCR grade water	25 ml
Total	**50 ml**

Preheat the buffer to 47°C in a water bath.

Component	Volume
10X SC Wash Buffer I	5 ml
PCR grade water	45 ml
Total	**50 ml**

Remove 10 ml of buffer to a separate 15 ml conical tube and preheat in a 47°C water bath for at least 5 minutes. Store the remaining buffer at room temperature.

Component	Volume
10X SC Wash Buffer II	5 ml
PCR grade water	45 ml
Total	**50 ml**

Store at room temperature after prepared.

Component	Volume
10X SC Wash Buffer III	5 ml
PCR grade water	45 ml
Total	**50 ml**

Store at room temperature after prepared.

Step 2. Prepare the Streptavidin Dynabeads

1. Warm the Streptavidin Dynabeads to room temperature for 30 minutes prior to use.
2. Vortex the Streptavidin Dynabeads for 1 minute to resuspend.
3. For each capture reaction, aliquot 100 µl of beads into a well of a 96-well PCR plate.
4. Place the PCR plate onto the MagnaBot II magnetic separation device.
5. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove supernatant and discard to waste.
6. Remove the PCR plate from the MagnaBot II device.
7. Add 200 µl of Bead Binding and Wash Buffer to each well of the 96-well plate and mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
8. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.
9. Remove the PCR plate from the MagnaBot II device.
10. Repeat Steps 2.7 - 2.9.
11. Add 100 µl of Bead Binding and Wash Buffer to each well of the 96-well plate and mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
12. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.

Step 3. Bind DNA to the Streptavidin Beads

1. Transfer each hybridization sample (approximately 15 µl) to a well of the 96-well plate containing the prepared Streptavidin Dynabeads.
2. Mix thoroughly by pipetting up and down 10 times.
3. Place the 96-well plate containing the sample and beads in a thermocycler and incubate for 45 minutes at 47°C. Mix the samples by vortexing at 15 minute intervals.

Step 4. Wash the Streptavidin Beads plus bound DNA

1. After the 45-minute incubation at 47°C, place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant to waste.
2. Add 100 µl of preheated (47°C) 1X SC Wash Buffer I to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
3. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.
4. Add 200 µl of 1X Stringent Wash Buffer heated to 47°C to each well of the 96-well plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
5. Place the PCR plate into a thermocylcer and incubate for 5 minutes at 47°C.
6. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.
7. Repeat Steps 4.4 - 4.6 for a total of two washes with the 1X Stringent Wash Buffer heated to 47°C.
8. Add 200 µl of room temperature 1X SC Wash Buffer I to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
9. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.
10. Add 200 µl of room temperature 1X SC Wash Buffer II to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
11. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.

12. Add 200 µl of room temperature 1X SC Wash Buffer III to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
13. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Streptavidin Dynabeads to the side of the tubes. Remove the supernatant and discard to waste.
14. Remove the plate from the MagnaBot II device and add 50 µl of PCR grade water to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Streptavidin Dynabeads into solution.
15. Store the beads plus captured samples at -15°C to -25°C or proceed to *Chapter 7. Captured DNA Amplification Using LM-PCR*.

There is no need to elute DNA off the beads. The beads plus captured DNA will be used as template in the LM-PCR as described in *Chapter 7*.

Appendix D. Limited Warranty

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