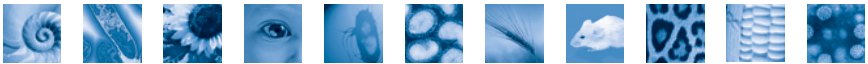




NimbleGen Arrays User's Guide

454 Optimized Sequence Capture Array Delivery

Version 1.1



For life science research only.
Not for use in diagnostic procedures.





NimbleGen Arrays User's Guide

*454 Optimized
Sequence Capture Array Delivery*

**For life science research only.
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Chapter 1. Before You Begin

This user's guide describes an optimized Sequence Capture method that permits direct sequencing of captured human genomic DNA fragments using the Genome Sequencer FLX Instrument and GS FLX Titanium reagents. This method is used to enrich a 100kb to 5Mb region of interest from human genomic DNA.

The 454 Optimized Sequence Capture method described in this user's guide is the preferred Sequence Capture method for targeted resequencing of DNA using the Genome Sequencer FLX Instrument. Each step has been optimized for robust, superior 454 sequencing performance with GS FLX Titanium General Library Preparation Kits. In particular, this improved procedure does not require the construction of an additional sequencing library after the sample is enriched using a NimbleGen Sequence Capture array. This significantly reduces labor and reagent costs. After a genomic DNA library is captured, eluted, and amplified, it is ready for emulsion PCR (emPCR amplification) using GS FLX Titanium LV or SV emPCR Kits (Roche Applied Science, Cat. Nos. 05233542001 (LV) and 05233615001(SV)).

This user's guide describes the library preparation method using the 454 GS FLX Titanium General Library Preparation Kit (Roche Applied Science, Cat. No. 05233747001), pre-capture LM-PCR, hybridization and washing of the NimbleGen Sequence Capture array, elution of the captured library, LM-PCR of captured samples, and quantitative PCR (qPCR) assays to assess capture success. The final chapter provides directions on preparing a captured library for sequencing with the Genome Sequencer FLX Instrument (Figure 1).

Required thermocycler reaction programs are detailed on page 14 (Chapter 3, Step 2.2), page 34 (Chapter 7, Step 2.2), and page 41 (Chapter 8, Table 1). Thermocyclers should be programmed before beginning this protocol.

What's New?

Version 1.1 includes these changes:

- Chapter 1, “Standard Laboratory Equipment” section (page 5):
 - Ordering information for a QIAvac 24 Vacuum Manifold and a vacuum pump is now provided. These devices are used in Chapter 6, Microarray Washing and Elution of Captured Samples.
 - Ordering information for the compressed gas nozzle has changed. The nozzle is now available from TeqCom instead of Roche NimbleGen.
- Chapter 1, “Oligonucleotide Consumables” section (page 8): The sequence for the Hybridization Enhancing 454 Ti-A oligonucleotide has been updated.
- Chapter 8, Table 1 (page 40): Table 1 now correctly shows that the Amplification step contains a cycle at 60°C instead of the Melting Curve step.
- Chapter 9: As a result of updates to the emPCR Kit and the protocol for the GS FLX Titanium Series, be aware of the following modification:
“Option 1: Emulsion Titration Assay” section, step 1 (page 48) includes updated amounts of diluted library for Tubes 1 - 4.

Note: 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/.

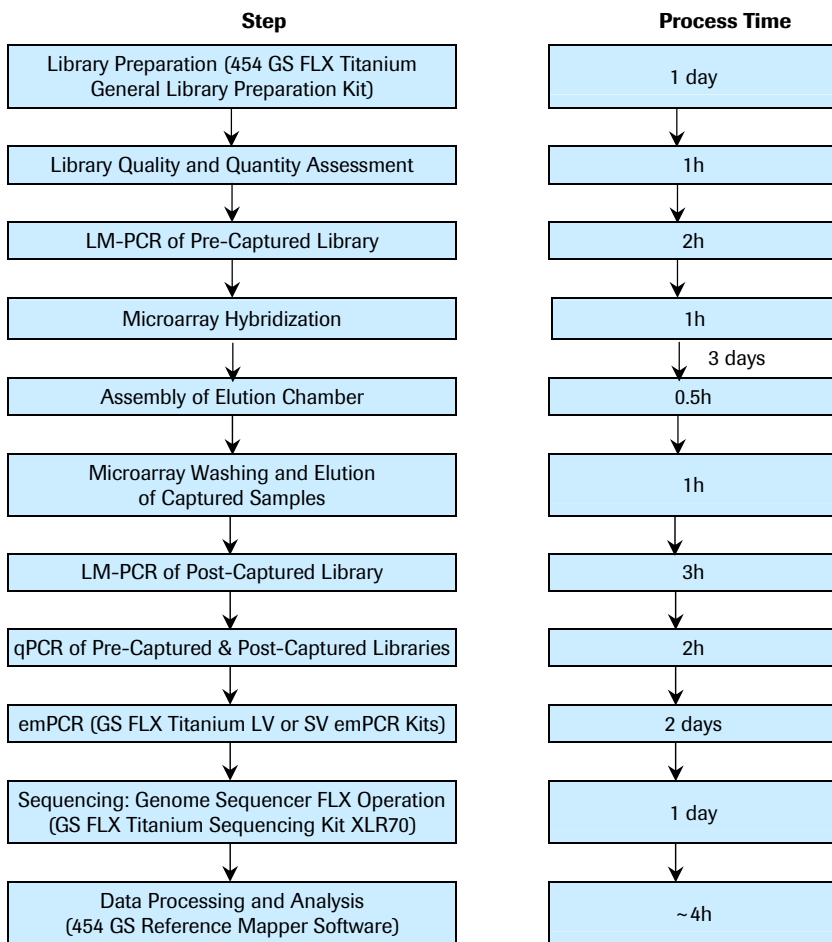


Figure 1: Workflow for NimbleGen 454 Optimized Sequence Capture Experiments. Steps in the process and estimated time for each step, based on the processing of one array, are shown in the boxes. Incubation times are indicated beneath the appropriate process times. Note that the procedures for the Data Processing and Analysis step are not detailed in this user's guide.

Components Supplied

Component	Description
NimbleGen Arrays	As ordered
NimbleGen Mixers	X1 mixer is provided with the 385K array
Mixer Port Seals	Port seals are provided with X1 mixers
Elution Chamber (ES1)	Disposable apparatus used with the NimbleGen Elution System for elution of captured DNA from 385K arrays
NimbleGen Arrays User's Guide: 454 Sequence Capture Array Delivery	Provided on the CD/DVD
CD/DVD	Design .gff and .bed files, visualization software, and user documentation are included

Microarray Storage

NimbleGen arrays are packaged with desiccant and can be stored at room temperature for use by the expiration date. Once the seal is broken, store NimbleGen arrays in a desiccator at room temperature until use.

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.

NimbleGen Equipment

Choose between the 4- or 12-bay NimbleGen Hybridization Systems.

Equipment	Supplier	Process Quantity	Item Number
NimbleGen Hybridization System 4*	Roche	4 slides	05223652001 (110V)
	NimbleGen		05223687001 (220V)
NimbleGen Hybridization System 12*	Roche	12 slides	05223679001 (110V)
	NimbleGen		05223695001 (220V)
NimbleGen Elution System	Roche NimbleGen	1 slide	05329752001

* NimbleGen Hybridization Systems include an accessory kit that contains Precision Mixer Alignment Tool (PMAT), Mixer Disassembly Tool, Mixer Brayer, System Verification Assemblies, replacement O-rings, and forceps.

Standard Laboratory Equipment

Equipment	Supplier	Item Number
Compressed Gas Nozzle	TeqCom	TA-N2-2000
Nitrogen or Argon Tank with 1 - 125psi regulator	VWR	55850-700
Desiccator	<i>Multiple Vendors</i>	
Heat Block (capable of temperatures to 120°C)	<i>Multiple Vendors</i> (Recommend the 6-bay digital hot block from VWR, #126210100)	
Microcentrifuge with Multiplate Adaptors (12,000 x g capability)	<i>Multiple Vendors</i>	
Microman M100 Pipette	Gilson	F148504
Spectrophotometer	NanoDrop	ND-1000

Equipment	Supplier	Item Number
Bioanalyzer 2100	Agilent	
LightCycler® 480 Instrument II	Roche Applied Science	05015243001 (384-well version) -or- 05015278001 (96-well version)
Genome Sequencer FLX Instrument	Roche Applied Science	04896548001
SpeedVac (1.5ml tubes)	Thermo Savant	
Thermocycler	<i>Multiple Vendors</i>	
Vortex Mixer	<i>Multiple Vendors</i>	
QIAvac 24 Vacuum Manifold	Qiagen	19413
Vacuum Pump	Qiagen	84010

Consumables & Accessories from Roche NimbleGen

Component	Package Size / Process Quantity	Item Number
NimbleGen X1 Mixer for 385K arrays (includes mixer port seals) ¹	10 mixers	05223725001
NimbleGen Sequence Capture Hybridization Kit	60 hybridizations for 385K arrays	05340721001
NimbleGen Sequence Capture Wash and Elution Kit	15 washes and elutions	05340730001
Elution Chamber ES1 ²	1 chamber & 1 gasket	05329809001
Elution System Retaining Ring (only order if a replacement is needed) ³	1 retaining ring	05329787001

- 1 NimbleGen X1 mixers, ordered at the same time as NimbleGen Sequence Capture 385K arrays, can be purchased as replacement parts. Mixers are disposable and intended for a single use.
- 2 A NimbleGen Elution Chamber ES1, included with a NimbleGen Sequence Capture 385K array, can be purchased separately. Elution Chambers and Gaskets are disposable and intended for a single use.
- 3 A NimbleGen Elution System Retaining Ring, included with a NimbleGen Elution System, can be ordered as a replacement part.

Contents of NimbleGen Sequence Capture Kits

Kit	Contents
NimbleGen Sequence Capture Hybridization Kit	<ul style="list-style-type: none"> ■ 2X SC Hybridization Buffer* ■ SC Hybridization Component A
NimbleGen Sequence Capture Wash and Elution Kit	<ul style="list-style-type: none"> ■ 2X Stringent Wash Buffer ■ 10X SC Wash Buffer I ■ 10X SC Wash Buffer II ■ 10X SC Wash Buffer III ■ Elution Reagent

* SC = Sequence Capture

Consumables from Roche Applied Science

Component	Supplier	Package Size	Item Number
Water (PCR grade)	Roche Applied Science	25ml (25 x 1ml)	03315932001
GS FLX Titanium General Library Preparation Kit	Roche Applied Science	10 library preparations	05233747001
GC-RICH PCR System, dNTPack	Roche Applied Science	100U	04743784001
COT Human DNA, Fluorometric Grade	Roche Applied Science	1mg/ml, 1ml	05480647001
LightCycler® 480 Multiwell Plate 384 (with sealing foils)	Roche Applied Science	5 x 10 plates	04729749001
LightCycler® 480 SYBR Green I Master (2X Mix)	Roche Applied Science	5 x 1ml	04707516001

Consumables from Other Vendors

Refer to the most recent version of *GS FLX Titanium General Library Preparation Method Manual* for additional standard laboratory equipment and materials required for use with the GS FLX Titanium General Library Preparation Kit (Roche Applied Science, Cat. No. 05233747001).

Component	Supplier	Package Size	Item Number
QIAquick PCR Purification Kit	Qiagen	250 reactions	28106
Water (reagent grade, ACS, nonsterile, type 1)	VWR	2.5 gallon	RC915025
Quant-iT Pico Green dsDNA Assay Kit	Invitrogen	1 kit	P7589
Compressed Nitrogen or Argon Gas (for cleaning array surface)*	<i>Multiple Vendors</i>		
CP100 Pipette Tips	Gilson	192 tips	F148414
		960 tips	F148314
Microfuge Tubes (1.5ml)	Ambion	500 tubes	12400
Pipettor Basin (multichannel, 100ml)	VWR	200 basins	89094-656

Component	Supplier	Package Size	Item Number
Disposable Forceps	Cole-Parmer	100 forceps	EW-06443-20
Washing Tubes	Fisher Scientific	1,000 tubes	05-553-4
Acetic Acid	Fisher Scientific	500ml	BP1185

* Roche NimbleGen recommends using a compressed gas nozzle to gently blow compressed nitrogen or argon gas across arrays to remove any dust or debris. The use of canned aerosol compressed air for this purpose is not recommended and could compromise array and data quality.

Oligonucleotide Consumables

Component	Supplier	Concentration	Sequence
Hybridization Enhancing 454 Ti-A ^{1,2}	IDT	1,000µM	5' - CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG/3ddc/ - 3'
Hybridization Enhancing 454 Ti-B ^{1,2}	IDT	1,000µM	5' - CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG/3ddc/ - 3'
LM-PCR 454 Ti-A ³	IDT	25µM	5' - CCA TCT CAT CCC TGC GTG TC - 3'
LM-PCR 454 Ti-B ³	IDT	25µM	5' - CCT ATC CCC TGT GTG CCT TG - 3'
qPCR oligo NSC-0237, forward ^{3,4}	IDT	2µM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'
qPCR oligo NSC-0237, reverse ^{3,4}	IDT	2µM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'
qPCR oligo NSC-0247, forward ^{3,4}	IDT	2µM	5' - CCC ACC GCC TTC GAC AT - 3'
qPCR oligo NSC-0247, reverse ^{3,4}	IDT	2µM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'
qPCR oligo NSC-0268, forward ^{3,4}	IDT	2µM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'
qPCR oligo NSC-0268, reverse ^{3,4}	IDT	2µM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'

Component	Supplier	Concentration	Sequence
qPCR oligo NSC-0272, forward ^{3, 4}	IDT	2µM	5' - CAG CCC CAG CTC AGG TAC AG - 3'
qPCR oligo NSC-0272, reverse ^{3, 4}	IDT	2µM	5' - ATG ATG CGA GTG CTG ATG ATG - 3'

- 1 Hybridization Enhancing 454 Ti-A and Ti-B must be resuspended in PCR-grade water.
- 2 /3ddc/ describes 3' Dideoxy-C modification for oligonucleotides produced by IDT.
- 3 Resuspend LM-PCR 454 Ti and qPCR oligos in PCR-grade water or TE buffer.
- 4 These oligos are used in qPCR analysis as described in Chapter 8.

Consumables for the Genome Sequencer FLX Instrument

Component	Supplier	Package Size	Item Number
GS FLX Titanium LV emPCR Kit (Lib-L)	Roche Applied Science	1 kit	05233542001
GS FLX Titanium emPCR Breaking Kit LV 12pc	Roche Applied Science	12 pieces	05233658001
GS FLX Titanium SV emPCR Kit (Lib-L)	Roche Applied Science	1 kit	05233615001
GS FLX Titanium Sequencing Kit XLR70	Roche Applied Science	1 kit	05233526001
GS FLX Titanium PicoTiterPlate Kit 70x75	Roche Applied Science	1 plate	05233682001
GS FLX Titanium emPCR Filters SV 64pc	Roche Applied Science	64 filters	05233674001
GS Reference Mapper Software	Roche Applied Science		

Technical Support

If you have technical questions, contact your Roche Account Manager or Roche Microarray Technical Support. Go to www.nimblegen.com/arraysupport for contact information.

Chapter 2. Library Preparation

Chapter 2 describes the library preparation method, how to assess the quality of the library and how to quantify the library for pre-capture LM-PCR.

Preparation of a GS FLX Titanium General DNA Library requires 3 - 5µg of genomic DNA for the double SPRI method or 5 - 10µg for the alternative gel cut method. Choose the appropriate fragment size selection method for your experiment and follow the protocol according to the option you choose. Refer to the *GS FLX Titanium General Library Preparation Method Manual*.

References

- GS FLX Titanium General Library Preparation Method Manual
 - Agilent RNA 6000 Pico Kit Guide
-

Step 1. DNA Sample Quality Requirement

Refer to the *GS FLX Titanium General Library Preparation Method Manual* for DNA sample quality criteria.

Step 2. Library Preparation

Construct the library following the procedure described in the *GS FLX Titanium General Library Preparation Method Manual*. Follow the protocol from section 3.1, DNA Fragmentation (Nebulization) through section 3.9, Single-Stranded DNA Library (sstDNA library) isolation.

Step 3. Library Quality Assessment and Quantification

1. Analyze 1 μ l of sstDNA library on Bioanalyzer RNA 6000 Pico chip.
2. Refer to the *GS FLX Titanium General Library Preparation Methods Manual* for guidelines for a successfully constructed sstDNA library.
3. Use the RNA 6000 Pico chip to estimate the concentration of the library. Make sure that the RNA area of the RNA ladder falls within the normal range of 100 - 150. If it is out of this range, run another RNA 6000 Pico chip or estimate the sample concentration by dividing the RNA area of the sample by 137. A typical RNA area for a 1 μ l ladder (1,000pg/ μ l) is 137.

Chapter 3. Pre-Capture Library Amplification by LM-PCR

This chapter describes how to amplify the GS FLX Titanium Series single-stranded DNA library that was prepared in Chapter 2, in preparation for microarray hybridization.

References

- GC-RICH PCR System, dNTPack Product Instructions (Roche Applied Science)
 - Thermocycler Manual
 - QIAquick Spin Handbook (Qiagen)
 - Agilent DNA 7500 Kit Guide
-

Sample Requirements

The amount of template required for *each* of the 5 reactions in the library amplification step should be ~3ng to avoid potential bias during amplification and to ensure sufficient yield. We estimate 3ng contains ~900 copies of the human genome. Amplification using smaller amounts of template per reaction, or fewer than 5 reactions, has not been tested.

Step 1. Prepare the LM-PCR Master Mix

1. Prepare the Pre-Capture LM-PCR Master Mix in a 1.5ml tube. The amount of each reagent needed for one capture (5 reactions) is listed below:

Pre-Capture LM-PCR Master Mix for 5 reactions (1 sample)

5X GC-RICH PCR reaction buffer (vial 2) ¹	50µl
25mM MgCl ₂ (vial 4) ¹	10µl
PCR Grade Nucleotide Mix (vial 6) ¹	5µl
25µM LM-PCR 454 Ti-A Oligo	5µl
25µM LM-PCR 454 Ti-B Oligo	5µl
PCR-grade water (vial 5) ¹	45µl ²
GC-RICH Enzyme Mix (vial 1) ¹	5µl
Total	125µl

- 1 These components are included in the Roche Applied Science GC-RICH PCR System, dNTPack (Cat. No. 04743784001).
 - 2 Adjust the volume of PCR-grade water if necessary to accommodate the volume of the template in each reaction (Steps 1.2 - 1.3).
2. Prepare 5 PCR tubes each containing ~3ng of template in a total volume of 25µl of PCR-grade water.
 3. Pipette 25µl of the LM-PCR Master Mix into each of the 5 tubes containing 25µl of template. Mix well by pipetting.

Step 2. Perform PCR Amplification

1. Place PCR tubes in the thermocycler.
2. Amplify samples using following Pre-Capture LM-PCR program:
 - Step 1: 4 minutes @ 94°C
 - Step 2: 0.5 minute @ 94°C
 - Step 3: 1 minute @ 58°C
 - Step 4: 1.5 minutes @ 68°C
 - Step 5: Repeat Steps 2-4, 11 times
 - Step 6: 3 minutes @ 68°C
 - Step 7: Hold @ 4°C
3. Store samples at 4°C until ready for cleanup.

Step 3. Clean up the Sample

1. Combine the 5 reactions for each sample into one 1.5ml microfuge tube (~250µl).
2. Follow the instructions provided with the QIAquick PCR Purification Kit (Qiagen) with slight modifications (listed below in Steps 3.3 - 3.8).
3. To each tube, add 1,250µl (5x volume) of Qiagen Buffer PBI. Mix well.
4. Pipette 800µl of the sample into a QIAquick PCR Purification Kit column. Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
5. Load the rest of the sample (~750µl). Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
6. Add 750µl of PE buffer to the column. Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
7. Place the QIAquick column in the same tube. Centrifuge at 10,000 x g for 1 minute.
8. Transfer the column to a clean 1.5ml microfuge tube. Add 50µl of PCR-grade water directly to the column matrix, changing tips between samples. Centrifuge at 10,000 x g for 1 minute to elute the DNA.

Important: It is critical that the amplified library is eluted with PCR-grade water rather than Buffer EB or 1X TE buffer.

Step 4. Determine the Concentration and Size Distribution of the Amplified Library

1. Measure A_{260}/A_{280} using the NanoDrop spectrophotometer to quantify the DNA concentration and determine sample quality.
2. Run 1µl of pre-capture LM-PCR product on a Bioanalyzer DNA 7500 chip to determine if the sample meets these requirements:
 - Pre-capture LM-PCR yield should be > 3.0µg.
 - The A_{260}/A_{280} should be 1.7 - 2.0.
 - The Bioanalyzer should indicate most library fragments falling between 350 - 1,000bp, with an average fragment length of between 500 - 800bp.

3. If the sample meets these requirements, proceed to hybridization (Chapter 4). If the sample does not meet these requirements, perform pre-capture LM-PCR with the remaining sstLibrary or re-construct the library.

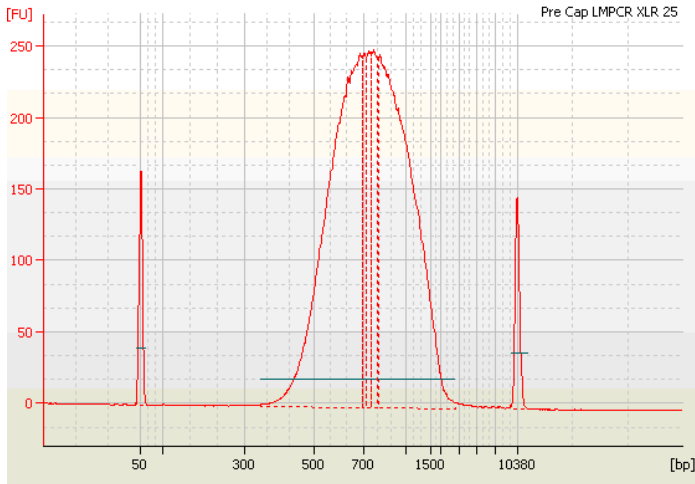


Figure 2: Example of a Successfully Amplified Pre-Capture LM-PCR Library Analyzed Using an Agilent 2100 Bioanalyzer DNA 7500 Chip

Chapter 4. Microarray Hybridization

Chapter 4 describes the NimbleGen protocol for sample hybridization. Be aware of the following:

- The hybridization protocol requires a NimbleGen Hybridization System. Refer to its user's guide for specific instructions on its use.
- The hybridization protocol requires adherence of a NimbleGen mixer to the microarray slide.

Reference

NimbleGen Hybridization System User's Guide (Roche NimbleGen)

Step 1. Prepare the Hybridization System

1. Set the Hybridization System to 42°C. With the cover closed, allow at least 3 hours for the heat block temperature to stabilize at 42°C.
2. Turn on one heat block to 95°C, one to 70°C, and one to 42°C. Let equilibrate.

Step 2. Prepare the Hybridization Cocktail

1. Add 100µg of COT Human DNA, Fluorometric Grade (Roche Applied Science) to 3µg of pre-capture LM-PCR amplified library.
2. Close each tube's lid and make a hole in the top of each tube's cap with an 18 - 20 gauge or smaller needle.

Note: The hole in the top of each tube's cap is a precaution to suppress contamination in the SpeedVac.

3. Dry the samples in a SpeedVac on high heat (60°C).

Note: This step may take 30 minutes or longer. To minimize drying time, dry the COT Human DNA ahead of time and then add the sample when ready.

Note: Denaturation of the DNA with high heat is not problematic after adaptor ligation because the hybridization utilizes single-stranded DNA.

4. Add 3.5µl of PCR-grade water to each sample to rehydrate.
5. Vortex the sample and centrifuge at maximum speed for 30 seconds.
6. Place the sample in a 70°C heat block for 10 minutes to fully solubilize the DNA.
7. Vortex the sample and centrifuge at maximum speed for 30 seconds.
8. Add 0.65µl of each Hybridization Enhancing 454 Ti-A and B (1.3µl total) to each sample.
9. Vortex the sample and centrifuge at maximum speed for 30 seconds.
10. Add to each sample:
 - 8µl of 2X SC Hybridization Buffer
 - 3.2µl of SC Hybridization Component A

The samples should now contain the components shown in the following table.

Component	
100µg COT Human DNA, Fluorometric Grade and 3.0µg pre-capture LM-PCR library sample	Dried down and resuspended in 3.5µl PCR-grade water
Hybridization enhancing oligo 454 Ti-A	0.65µl
Hybridization enhancing oligo 454 Ti-B	0.65µl
2X SC Hybridization Buffer	8µl
SC Hybridization Component A	3.2µl
Total	16µl

11. Vortex samples and centrifuge at maximum speed for 30 seconds.
12. Place each sample in a 95°C heat block for 10 minutes to denature the DNA.
13. Centrifuge samples at maximum speed for 30 seconds and place at 42°C until ready for hybridization (hybridize within 15 minutes of denaturation).

Step 3. Prepare Mixers

1. Remove the NimbleGen X1 mixer from its package.

Note: For best results, use a compressed gas nozzle to blow compressed gas across the mixer and slide to remove any dust or debris. Load samples within 30 minutes of opening the vacuum-packaged mixer to prevent the formation of bubbles during loading and/or hybridization.

2. Position the Precision Mixer Alignment Tool (PMAT) with the hinge on the left and then open it.
3. Snap the mixer onto the two alignment pins on the lid of the PMAT with the tab end of the mixer toward the inside hinge and the mixer's adhesive gasket facing outward (Figure 3).
4. Place the slide in the base of the PMAT while pushing back the plastic spring so that the barcode is on the right and facing up and the corner of the slide sits against the plastic spring. Remove your thumb and make sure the spring is engaging the corner of the slide and the entire slide is registered to the edge of the PMAT to the rightmost and closest to you. In addition, be sure that the slide is lying flat against the PMAT.

Note: Take care to align the slide correctly in the PMAT.

5. Gently blow compressed nitrogen or argon gas across the mixer and slide.
6. Remove the backing from the adhesive gasket using forceps and close the lid of the PMAT so that the gasket makes contact with the slide.

7. Lift the lid by grasping the long edges of the PMAT while simultaneously applying pressure with a finger through the window in the lid of the PMAT to free the mixer-slide assembly from the pins.

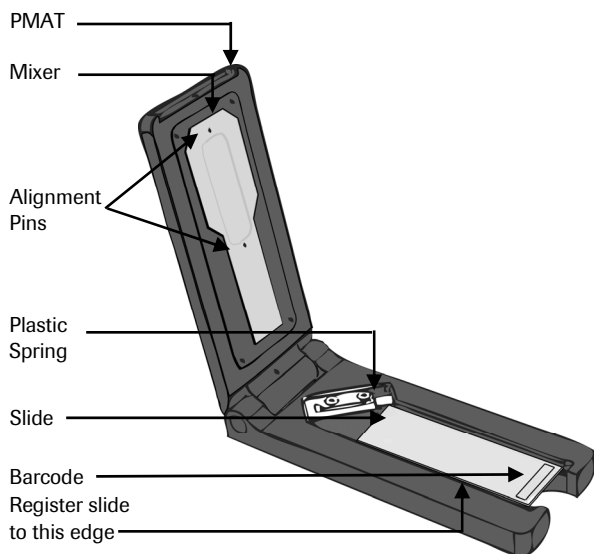


Figure 3: PMAT with Mixer and Slide

8. Remove the mixer-slide assembly from the PMAT. Place the mixer-slide assembly on a clean, smooth, dark, flat surface.
Note: Place the mixer-slide assembly on the back of a 42°C heat block to facilitate adhesion of the mixer to the slide.
9. Rub the Mixer Brayer over the mixer with moderate pressure to adhere the adhesive gasket and remove any bubbles. Start in the center of the array and rub outwards. The adhesive gasket will become clear when fully adhered to both surfaces.
10. Repeat Steps 3.1 - 3.9 for all slides to hybridize.
11. Place each mixer-slide assembly in the slide bay of the Hybridization System.

Step 4. Load and Hybridize Samples

1. Confirm that each sample is matched with the appropriate microarray.

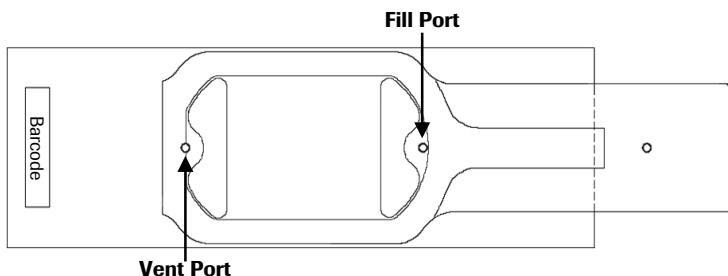


Figure 4: X1 Mixer and Slide

2. Use a Microman 100 Pipette with CP100 tips (Gilson) to pipette as much of the sample as necessary to completely fill the mixer chamber without introducing any bubbles (~15 - 16 μ l) into the fill port. After loading, make sure there are no bubbles in the mixer chamber. If there are, gently massage bubbles to either of the ends of the mixer chamber or to the sides. Pipette any extra sample into the port (where there is no bubble) and remove bubbles through the opposite port.
3. Dry any exposed sample from the fill and vent ports using a clean tissue or cotton swab for each array to avoid cross-contamination.
4. Adhere port seals to cover the fill and vent ports on the mixer. Press on both the vent and fill port stickers at the same time to ensure equal pressure.
5. Close the bay clamp until locked (you should hear a click).
6. Turn on the Mixing Panel and set the mix mode to B on the Hybridization System.
7. Confirm the system recognizes the slide in each occupied bay.

- 8.** Approximately 5 - 10 minutes after starting the Hybridization System:
 - Ensure that the mix mode is set to B.
 - Ensure that the green light is displayed for all occupied stations.
 - Ensure that the temperature reads 42°C.
- 9.** Hybridize samples at 42°C for at least 64 hours (no more than 72 hours).

Chapter 5. Assembly of Elution Chamber

Chapter 5 describes the processes for assembly of the Elution Chamber. It is essential that the Elution Chambers are assembled before beginning Chapter 6, Microarray Washing and Elution of Captured Samples. Elution Chambers and Gaskets are disposable. Use once and discard to avoid cross-contamination between samples.

Step 1. Inspect the Elution Chamber Components

1. While wearing gloves, in a workplace in which GS FLX Titanium Series adapted genomic DNA has **not** been manipulated (e.g. laminar fume hood), remove the clear polycarbonate Elution Insert from its plastic bag.

Note: Elution Chambers should not be exposed to 454 adaptor ligated libraries or LM-PCR amplified DNA since such molecules could cross-contaminate samples.

2. Remove one Elution System Gasket for each Elution Chamber to be assembled from the shipping bag.
3. Visually examine the Elution System Gasket to make sure it is intact.

Note: The Elution System Gasket has a square cross-sectional shape with a tiny groove on each face of the square. Visual examination of the groove during assembly identifies twists in the Elution System Gasket during assembly.

Step 2. Assemble the Elution Chamber

1. Wearing gloves, insert the Elution System Gasket into the oblong channel of the molded Elution Insert, starting with the two straight portions of the channel.

2. While holding down the center portion of the Elution Insert, work the Elution System Gasket into one side of the rounded part of the channel (Figure 5).
3. Fit the Elution System Gasket into the remaining rounded part of the channel while still holding down the center portion of the Elution Insert (Figure 5).
4. Gently press the Elution System Gasket down into the channel to ensure proper, uniform Elution Chamber assembly (Figure 5).

Note: Do not twist the Elution System Gasket in any way. Visual inspection should reveal the same face of the square facing up. Trace the path of the tiny groove on the Elution System Gasket once it is in the channel. A twist in the Elution System Gasket could result in a poor seal to the array face resulting in a leaky elution.

Important: The Elution System Gasket needs to be evenly distributed within the Elution Insert channel to be sure that its entire length is accommodated.

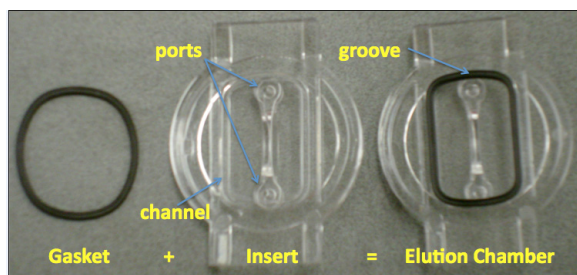


Figure 5: Assembly of an Elution Chamber ES1 Using the Elution System Gasket and the Elution Insert

Chapter 6. Microarray Washing and Elution of Captured Samples

Chapter 6 describes the processes for single tube microarray washing and elution of the captured gDNA samples for batches of 6 slides or fewer. The number of microarrays processed in one batch should not exceed the number of available Elution Systems. These processes are prone to sample cross-contamination, which can be amplified by downstream PCR steps. Take appropriate precautions. Prepare single-use wash tubes for each sample. Discard tubes after one use.

Note: It is extremely important that the water bath temperature is closely monitored and remains at 47.5°C ($\pm 1.0^\circ\text{C}$). A high quality thermometer placed in the water bath is recommended since the displayed temperatures on many water baths are often imprecise.

Note: Equilibrate buffers at 47.5°C for at least 2 hours before washing microarray.

Step 1. Prepare for Wash and Elution

1. Dilute 10X SC Wash Buffers (I, II, and III) and 2X Stringent Wash Buffer to 1X working solutions.
2. For each slide, prepare the following SC Wash Buffers:
 - Two washing tubes with 32ml of Stringent Wash Buffer heated to 47.5°C in a water bath
 - One washing tube with 32ml of SC Wash Buffer I at room temperature
 - Two washing tubes with 32ml of SC Wash Buffer II: one at 47.5°C and one at room temperature
 - One disassembly basin (100ml multichannel pipettor basin) with 100ml SC Wash Buffer II heated to 47.5°C

Note: Recent array hybridization data suggest that significant inter-array variation is caused by temperature fluctuations that can occur during the transfer between the 42°C NimbleGen Hybridization System and the 47.5°C SC Wash Buffer II in the disassembly basin. Therefore, this step could be among the most critical in the procedure. Make sure arrays are kept at a constant 42°C throughout the hybridization step and that they are immediately transferred, one at a time, to wash buffer in the disassembly basin temperature maintained at 47.5°C for mixer disassembly.

- One washing tube with 32ml of SC Wash Buffer III at room temperature
3. Prepare 125mM sodium hydroxide (NaOH). The following volumes are appropriate for processing single arrays. Adjust volumes accordingly if processing multiple arrays:
 - Mix 493.75µl PCR-grade water and 6.25µl 10M NaOH.
 - Vortex to mix, spin down briefly, and store at room temperature until use.
 4. For each array to be eluted, fill a separate 1.5ml tube with 425µl of 125mM NaOH at room temperature.
 5. Prepare 20% acetic acid:
 - 400µl PCR-grade water
 - 100µl acetic acid, glacial
 - Vortex to mix, spin down briefly, and store at room temperature until use.

Note: Prepare fresh 125mM NaOH and 20% acetic acid solutions each time prior to use.

Step 2. Disassemble and Wash Microarrays

1. Double-glove both hands (double-gloving facilitates easy removal of the outer gloves between each sample). Change gloves between each sample to avoid cross-contamination.

2. Remove the slide from the 42°C Hybridization System and place the slide directly into the disassembly basin containing 100ml of SC Wash Buffer II heated to 47.5°C.
3. Wait ~10 seconds for the temperature to equilibrate. Carefully peel the mixer off the slide.

Note: Handle the slide only by the edges and portions exposed before mixer removal. Never touch the surface of the slide inside the mixer area. Agitate the slide up and down for 5 seconds in SC Wash Buffer II.

Important: The mixer is extremely flexible. Peel the mixer off slowly to avoid breaking the slide.

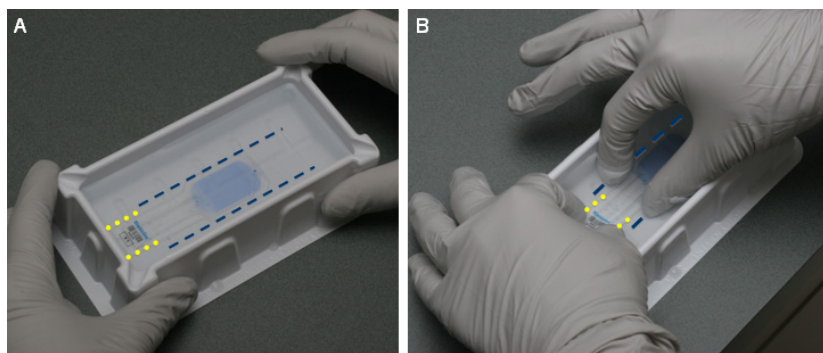


Figure 6: Disassembly of X1 Mixer from Slide. (A) Note the front edge of the mixer (dotted lines) and the edges of the slide (dashed lines). (B) Depiction of how the edges of the slide and the edges of the mixer should be handled to properly remove the mixer from the slide. For visualization purposes only, this figure depicts the mixing chamber of the mixer (both A and B) loaded with dye.

4. Transfer the slide to a wash tube containing 47.5°C SC Wash Buffer II, fix the cap, and invert the tube 10 times at a rate of 1 inversion per second.
5. Remove the outer gloves and replace with new gloves.
6. Repeat Steps 2.2 - 2.5 until all slides have been disassembled, using new disassembly basins, wash tubes, and SC Wash Buffers for each slide to be disassembled. To ensure that the SC Wash Buffer II does not cool below 47.5°C, we recommend that disassembly and washing of multiple slides are performed in batches of ≤ 6 .

- 7.** Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing SC Wash Buffer II. Place slides in new wash tubes containing 32ml of 47.5°C Stringent Wash Buffer (1 slide per tube).
- 8.** Invert the tubes 10 times at a rate of 1 inversion per second. Place in the tube rack for 5 minutes at 47.5°C.
- 9.** Remove the tubes from 47.5°C incubator. Invert the tubes 10 times at a rate of 1 inversion per second.
- 10.** Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Stringent Wash Buffer at 47.5°C.
 - Place slides in new wash tubes containing 32ml of 47.5°C Stringent Wash Buffer (1 slide per tube).
- 11.** Invert the tubes 10 times at a rate of 1 inversion per second. Place in the tube rack for 5 minutes at 47.5°C.
- 12.** Remove the tubes from 47.5°C incubator and invert the tubes 10 times at a rate of 1 inversion per second.
- 13.** Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Stringent Wash Buffer at 47.5°C.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer I at room temperature (1 slide per tube).
- 14.** Invert the tubes at a rate of 1 inversion per second for 2 minutes.
- 15.** Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Wash I.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer II at room temperature (1 slide per tube).
- 16.** Invert the tubes at a rate of 1 inversion per second for 1 minute.
- 17.** Using new, disposable forceps for each slide to be handled, quickly and carefully remove the slides from the wash tubes containing SC Wash Buffer II.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer III at room temperature (1 slide per tube).

18. Invert the tubes 10 times at a rate of 1 inversion per second.

Important: Avoid prolonged exposure of the slide in SC Wash Buffer III before elution.

Step 3. Elute the DNA

Roche NimbleGen recommends that the following procedure be performed as quickly as possible.

Note: Perform the following steps at room temperature. Place the Elution System in a heat block that is turned off or on a bench top. If multiple arrays are being eluted simultaneously, assemble each one in the Elution System as quickly as possible to prevent DNA from eluting into SC Wash Buffer III.

1. Remove the slides from the wash tubes using your gloved fingers. Gently tap the end of the slide on a clean tissue to remove as much excess buffer as possible.

Note: Be careful not to tap too hard and break the slide.

2. Wipe the *back* side of the slide with a clean tissue (the *front* side of the slide is the side on which you can read the barcode numbers in the correct orientation). Place the slide *front* side up within the Elution System at room temperature (Figure 7).

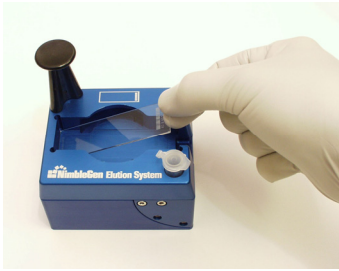


Figure 7: Placing a Slide into the Elution System

3. Place the disposable Elution Chamber on top of the slide with the Elution System Gasket facing down (Figure 8).



Figure 8: Placing the Elution Chamber in the Elution System

4. Place an Elution System Retaining Ring over the Elution Chamber and lock into place (Figure 9).



Figure 9: Placing and Locking the Elution System Retaining Ring

5. Using its handle, tilt the top of the Elution System until it locks into the upright position. Slowly pipette $\sim 425\mu\text{l}$ of the freshly made 125mM NaOH into the bottom (filling) port of the Elution Chamber (Figure 10).

Important: Do not tilt the Elution System without a pipette tip placed in the filling port or the 125mM NaOH will leak out (Figure 10).

Note: Pipette the 125mM NaOH solution slowly so it does not come out the top (vent) port.

Return the Elution System to the horizontal position. Return any excess 125mM NaOH that does not fit in the Elution Chamber to the original 1.5ml tube.

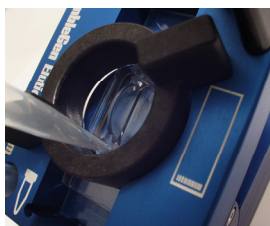


Figure 10: Tilting the Elution System and Loading Sample into the Elution Chamber

6. Incubate at room temperature for 10 minutes.
7. After incubation, pipette eluted DNA from the Elution Chamber and place into a 1.5ml tube containing any extra volume of 125mM NaOH that did not fit into the Elution Chamber.

Disassemble and discard the Elution Chamber.

Note: The Elution System can be tilted to an upright position, and a pipette can be used to remove any remaining volume from the slide to transfer it to the 1.5ml tube containing the eluted DNA.

Note: Do not re-use arrays or Elution Chambers because adaptor-ligated DNA will be carried over from the previous experiment. Observe all national, regional, and local regulations for proper disposal of microarrays and Elution Chambers.

8. Add 20% acetic acid solution to the Qiagen Buffer PBI in a separate 1.5ml tube. For each array processed, mix 16 μ l 20% acetic acid with 500 μ l Buffer PBI. Pipette up and down 10 times to mix.
9. Add 20% acetic acid/Buffer PBI mixture (~516 μ l) to eluted samples (~450 μ l). Invert the tube to mix.

Note: The color of the eluate/acetic acid/PBI should be yellow. If the color is purple, add 20% acetic acid, 1 μ l at a time, to the DNA mixture until the color changes to yellow.

10. Prepare the vacuum manifold and MinElute column following the supplier's instructions. Transfer the DNA/PBI/acetic acid mixture to a single MinElute column on the vacuum manifold. Draw the solution through the column and release the vacuum. Note that the maximum capacity of a column is 750 μ l, so multiple loadings will be required to transfer the entire volume of each sample to a single MinElute column/captured sample.

Note: Roche NimbleGen strongly recommends the use of a vacuum manifold, instead of a microcentrifuge, for the purification of DNA using the Qiagen MinElute columns. The use of a vacuum manifold may decrease the chance of contamination between samples when multiple samples are purified simultaneously. Refer to the Qiagen MinElute Handbook for the following steps.

- 11.** Add 750µl of Buffer PE (containing EtOH per the manufacture's instructions) to the column on the vacuum manifold. Draw the solution through the column and release the vacuum.
- 12.** Place the MinElute column into a 1.5ml tube or 2ml collection tube and centrifuge at maximum speed for 1 minute to remove any residual Buffer PE. Discard the flow-through.
- 13.** Place the MinElute column in a clean 1.5ml tube. Add 50µl Buffer EB to the center of the column, wait 1 minute, and centrifuge at maximum speed for 1 minute.
- 14.** Proceed to Chapter 7, LM-PCR on Captured Samples, or keep the eluate at -20°C until use.

Chapter 7. LM-PCR on Captured Samples

Chapter 7 describes the amplification of eluted, captured DNA samples using LM-PCR.

References

- GC-RICH PCR System, dNTPack Product Instructions (Roche Applied Science)
- Thermocycler Manual
- QIAquick Spin Handbook (Qiagen)
- Agilent DNA 7500 Kit Guide

Step 1. Prepare the LM-PCR Master Mix

1. Prepare the LM-PCR Master Mix in a 1.5ml tube. The amount of each reagent needed for 10 reactions (1 captured sample) is listed below:

Post-Capture LM-PCR Master Mix for 10 reactions (1 captured sample)	
5X GC-RICH PCR reaction buffer (vial 2)*	100µl
25mM MgCl ₂ (vial 4)*	20µl
PCR Grade Nucleotide Mix (vial 6)*	10µl
25µM LM-PCR 454 Ti-A Oligo	10µl
25µM LM-PCR 454 Ti-B Oligo	10µl
PCR Grade Water (vial 5)*	300µl
GC-RICH Enzyme Mix (vial 1)*	10µl
Total	460µl

* These components are included in the GC-RICH PCR System, dNTPack (Roche Applied Science)

2. Prepare 10 PCR reactions, each containing 4 μ l of captured DNA library, eluted in Chapter 6, as template. This number of PCR reactions is necessary to minimize possible amplification bias and ensure sufficient yield for downstream procedures.
 3. Pipette 46 μ l of LM-PCR Master Mix into each of the 10 reactions. Mix by pipetting up and down.
-

Step 2. Perform PCR Amplification

1. Place PCR tubes in the thermocycler.
 2. Amplify samples using following Post-Capture LM-PCR program:
 - Step 1: 4 minutes @ 94°C
 - Step 2: 0.5 minute @ 94°C
 - Step 3: 1 minute @ 58°C
 - Step 4: 1.5 minutes @ 68°C
 - Step 5: repeat Steps 2-4, 19 times
 - Step 6: 3 minutes @ 68°C
 - Step 7: Hold @ 4°C
 3. Store reactions at 4°C until ready for purification.
-

Step 3. Purify the Sample from the LM-PCR Reactions

1. Combine the 10 reactions for each sample into two 1.5ml microfuge tubes (5 reactions (~250 μ l)/tube, 2 tubes).
2. Follow the instructions provided with the QIAquick PCR Purification Kit (Qiagen) with slight modifications (listed below in Steps 3.3 - 3.10).
3. To each tube, add 1,250 μ l (5x volume) of Qiagen Buffer PBI. Mix well.
4. Pipette ~750 μ l of sample to a QIAquick column. Centrifuge at $\geq 10,000 \times g$ for 1 minute. Discard the flow-through.
5. Load the rest of the sample (~750 μ l) to the same QIAquick column. Centrifuge at $\geq 10,000 \times g$ for 1 minute. Discard the flow-through.
6. Add 750 μ l of buffer PE to the column. Centrifuge at $\geq 10,000 \times g$ for 1 minute to wash. Discard the flow-through.

7. Place the QIAquick column in the same tube. Centrifuge at $\geq 10,000 \times g$ for 1 minute.
8. Rotate the columns 180° and spin 30 seconds to remove residual ethanol in the Buffer PE.
9. Transfer the column to a clean 1.5ml microfuge tube. Add $50\mu\text{l}$ of TE buffer (from the GS FLX Titanium General Library Preparation Kit) directly to the column matrix. Let the column stand for 1 minute. Centrifuge at $\geq 10,000 \times g$ for 1 minute to elute the DNA.
10. Combine the two $50\mu\text{l}$ eluates per sample ($100\mu\text{l}$ total).

Step 4. Determine the Concentration, Size Distribution, and Quality of the Sample

1. Analyze $1\mu\text{l}$ of the sample (post-capture LM-PCR product) using a Bioanalyzer DNA 7500 chip and measure A_{260}/A_{280} using the NanoDrop spectrophotometer to quantify the concentration of DNA and to determine sample quality. Samples should exhibit the following characteristics:
 - A_{260}/A_{280} : 1.7 - 2.0
 - LM-PCR yield $> 1.0\mu\text{g}$
 - No visible primer dimer peak
 - Average fragment length between 500 - 800bp

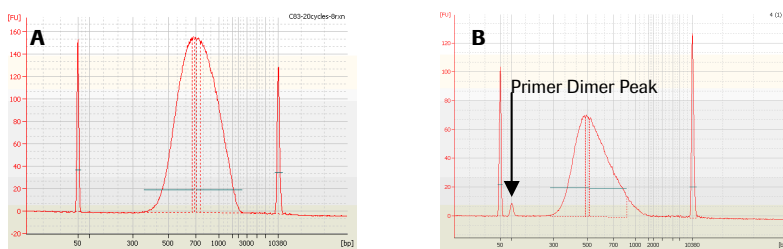


Figure 11: Agilent 2100 Bioanalyzer DNA 7500 Electropherogram of (A) Successfully Amplified Post-Capture LM-PCR Library and (B) Post-Capture LM-PCR Library with a Visible Primer Dimer Peak. Note that no primer dimer is evident in panel A. If primer dimer is observed (panel B), proceed with Step 4.3 in this chapter (below) for additional purification. For optimal 454 sequencing performance, fragments smaller than 350bp and larger than 1,000bp should constitute less than 10% of the total library mass.

2. If samples meet the requirements, proceed to Chapter 8, qPCR on LM-PCR Amplified Samples.

If samples do not meet the A_{260}/A_{280} requirement, purify the sample again using a second column.

3. If a primer dimer peak is observed, adjust sample volume to 100 μ l and add the amount of AMPure beads appropriate for the double SPRI method as described in the *GS FLX Titanium General Library Preparation Manual*, section 3.2.2, determined by the calibration of the lot number in use. Follow the protocol up to 3.2.2 Step 8, except to add 100 μ l of TE buffer (from the GS FLX Titanium General Library Preparation Kit) instead of 24 μ l. Proceed to 3.2.2, Step 9. Re-analyze 1 μ l of purified sample on a Bioanalyzer DNA 7500 chip to ensure no primer dimers are evident.

Chapter 8. qPCR on LM-PCR Amplified Samples

Chapter 8 describes the qPCR assays used to estimate relative fold-enrichment. These assays are an inexpensive way to determine whether the capture was successful prior to sequencing.

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 on every NimbleGen Human Sequence Capture array. Comparison by qPCR of the relative DNA concentrations of these control loci in pre-captured and captured samples allows for the estimation of enrichment of a capture target before committing sample libraries 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.

Note: A “DNA sample” in this chapter is defined as one pre-captured LM-PCR product (Chapter 3) and the matching captured LM-PCR product (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 captured DNA sample will require 24 individual qPCR reactions for analysis. One 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 NTC and positive controls will each require 12 additional qPCR reactions (refer to Figure 12).

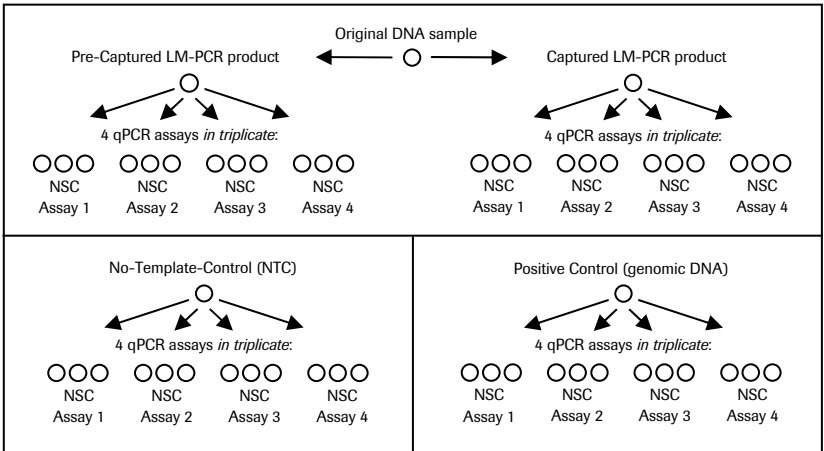


Figure 12: qPCR Experimental Overview

- The number of reaction wells required to perform a complete control locus qPCR analysis is determined by the formula, $W = 24 \times (S + 1)$, where W is the total number of wells required and S is the number of original DNA samples in the experiment (one per array). For example, analyzing one captured DNA sample, one NTC, and one positive control with 4 NSC assays performed in triplicate requires 48 individual qPCR reactions, $W = 24 \times (1 + 1)$. Analyzing two captured DNA samples requires 72 qPCR reactions, $W = 24 \times (2 + 1)$, etc.

- Dilute the NSC assay forward and reverse primers to 2 μ M (see Table 2, Step 1.6 on the next page).
- Dilute sufficient amounts of pre-captured and captured LM-PCR products to a concentration of 5ng/ μ l in PCR-grade water for use as qPCR templates (~100 μ l each).

Note: These LM-PCR products are used as templates in the control locus qPCR reactions. The initial concentration of these products was previously determined (Chapter 3, Step 4.1 and Chapter 7, Step 4.1).

*Important - for users of multi-array sets: If you are performing Sequence Capture using a multi-array set, and decided to pool your multiple library preparations to increase their uniformity prior to hybridization (Appendix A), you also will have pooled the pre-captured LM-PCR products into a single tube. Note that captured LM-PCR products should **not** have been pooled.*

- Add the following for a 15 μ l NSC assay (for larger volume reactions, adjust the reagent amounts proportionally):

qPCR Master Mix for 1 reaction	
PCR-grade water	5.9 μ l
2 μ M NSC Assay forward primer	0.3 μ l
2 μ M NSC Assay reverse primer	0.3 μ l
2X SYBR Green Master	7.5 μ l
5ng/ μ l template (pre-captured or captured LM-PCR; use genomic DNA for positive control -or- 1 μ l of PCR-grade water for negative control)	1 μ l
Total	15μl

- Program the qPCR instrument using the conditions specified in Table 1.

Note: 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			Ramp	Acquisitions (per °C)	Acquisition mode
			Target (°C)	Hold (hh:mm:ss)	Rate (°C/s)		
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 validated on NimbleGen Sequence Capture arrays using human captured DNA. The use of these assays for analyzing captured DNA from other species has not been tested and is not currently supported by Roche NimbleGen.

NSC qPCR Assay Name	Primer Sequences (5' → 3')	T _m (°C)	Product Length	qPCR Efficiency (£)
NSC-0237	F: CGCATTCTCATCCAGTATG R: AAAGGACTTGGTCGACAGTTCAG	81.15	80bp	1.84
NSC-0247	F: CCCACCGCCTTCGACAT R: CTGCTTACTGTGGGCTCTTG	81.03	74bp	1.80
NSC-0268	F: CTCGCTTAACCAGACTCATCTACTGT R: ACTTGGCTCAGCTGTATGAAGGT	78.99	75bp	1.78
NSC-0272	F: CAGCCCCAGCTCAGGTACAG R: ATGATGCGAGTGCTGATGATG	82.23	71bp	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 NimbleGen Sequence Capture arrays 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 pre-captured LM-PCR templates, captured LM-PCR templates, NTCs, 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 Basic software 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 pre-captured and captured DNA samples.
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.

Note: 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.

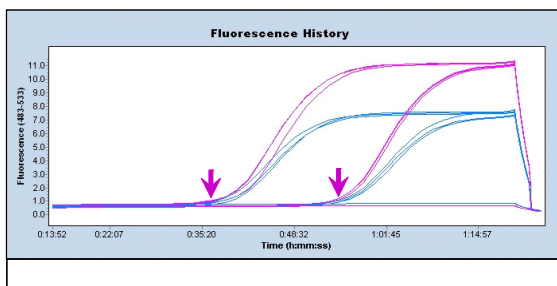


Figure 13: Example of Sequence Capture qPCR Data for Two NSC Assays Generated Using the LightCycler® 480 Instrument II. In a successful experiment, the C_p values from qPCR of capture LM-PCR templates (CAP) will be significantly lower than C_p values from pre-capture templates (PRE) for all assays.

Step 3. Analyze Data

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

Note: Replicate C_p values obtained from qPCR of post-LM-PCR samples should be similar. If the calculated standard deviation for 3 replicate C_p values is > 0.2 for a qPCR assay, consider repeating that assay to obtain more consistent results.

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

Note: Primer dimer amplification can occasionally produce an increase in fluorescence signal in an NTC reaction in the absence of target template molecules, but this typically occurs very late in the run (i.e. C_p 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 an NTC 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 C_p value determinations.

3. For each different sample and NSC assay combination, subtract the average Cp value measured for the captured LM-PCR template from the average Cp value measured for the matching pre-capture LM-PCR template. This value is the delta-Cp. A successfully enriched captured sample should generate a lower Cp value than its matched pre-captured sample. 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 $E^{\text{delta-Cp}}$.

Note: 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 NCS 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 $E = 10^{(-1/\text{slope})}$. 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 pre-captured, post-LM-PCR sample = 28.3, 28.5, 28.4
- Replicate Cp values for captured, post-LM-PCR sample = 17.5, 17.3, 17.7
 - Average $Cp_{\text{pre-captured}} = 28.4$
 - Average $Cp_{\text{captured}} = 17.5$
 - Delta-Cp = 10.9
 - Fold enrichment ($E^{\text{delta-Cp}} = (1.78)^{10.9} = 537$)

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

- Replicate C_p values for pre-captured, post-LM-PCR sample = 28.0, 28.2, 28.1
- Replicate C_p values for captured, post-LM-PCR sample = 18.2, 18.1, 18.0
 - Average $C_{p_{\text{pre-captured}}} = 28.1$
 - Average $C_{p_{\text{captured}}} = 18.1$
 - Delta- $C_p = 10$
 - Fold enrichment ($E^{\text{delta-}C_p}) = (1.93)^{10} = 717$

Note: Average C_p values for no-template-control (NTC) assays should be negligible, or they may indicate the presence of cross-contamination among wells or reagent contamination. If this is observed, repeat the qPCR experiment.

Note: Average C_p values for positive control (genomic DNA) assays should be similar (within $\sim 1 C_p$) to the average C_p values obtained for non-captured, post-LM-PCR samples.

Data Analysis Considerations

Careful consideration and data interpretation are necessary when deciding whether to sequence captured samples. 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 $\pm 50\%$ (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 whether to optimize NimbleGen Sequence Capture designs iteratively. Then 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.

Chapter 9. Preparation for emPCR Amplification and for Sequencing

Chapter 9 describes how to prepare for emPCR amplification using the GS FLX Titanium reagents and sequencing using a Genome Sequencer FLX Instrument. The captured sample has been amplified and is now ready for emPCR amplification using the GS FLX Titanium reagents.

References

- Invitrogen Quant-iT PicoGreen dsDNA Assay Kit Manual (June 10, 2008)
 - GS FLX Titanium General Library Preparation Method Manual (April 2009)
 - GS FLX Titanium emPCR Method Manual - Lib-L LV (January 2010)
 - GS FLX Titanium emPCR Method Manual - Lib-L MV (January 2010)
 - GS FLX Titanium emPCR Method Manual - Lib-L SV (January 2010)
 - GS FLX Titanium Sequencing Method Manual (January 2010)
-

Step 1. Library Quantification by Fluorometry

NimbleGen Sequence Capture products generated using the 454 Optimized Sequence Capture protocol are *double-stranded* and contain *Titanium A/B adapters* following the post-capture LM-PCR amplification and purification steps.

1. Refer to the concentration of the LM-PCR amplified, captured sample that was measured in Chapter 7, Step 4.1 of this user's guide.
2. Dilute the sample so that the concentration is within the range of the PicoGreen standards as outlined in the *Invitrogen Quant-iT PicoGreen dsDNA Assay Kit Manual*.
3. Prepare the PicoGreen reaction according to the manufacturer's instructions and measure the absorbance of the sample.

4. Calculate the library concentration equivalence in molecules/ μl . For details on the calculation, refer to the *GS FLX Titanium General Library Preparation Method Manual*, section 3.10.2, Step 1 with the following modification. Use 656.6 g/mole for the molecular mass of an average nucleotide pair in double-stranded DNA instead of 328.3 g/mole for each nucleotide in single-stranded DNA. For dilution and storage, refer to the *GS FLX Titanium General Library Preparation Method Manual*, section 3.10.2, Steps 2 and 3.

Step 2. Determination of the Amount of Library to Use in emPCR Amplification

Option 1: Emulsion Titration Assay

Refer to the *GS FLX Titanium General Library Preparation Method Manual*, section 3.10.3.1.

1. Dilute samples to 1×10^5 molecules/ μl . Set up 4 single tube emPCR amplification reactions as indicated below. Perform the rest of the emPCR amplification procedure using the “Small Volume Emulsion” protocol described in the *GS FLX Titanium emPCR Method Manual*.
 - Tube 1: 3.6 μl of diluted DNA library (= 0.15 molecule/bead)
 - Tube 2: 7.2 μl of diluted DNA library (= 0.30 molecule/bead)
 - Tube 3: 10.8 μl of diluted DNA library (= 0.45 molecule/bead)
 - Tube 4: 14.4 μl of diluted DNA library (= 0.60 molecule/bead)
2. Count and calculate percentage of bead enrichment as described in 3.7.6. Use the amount of input DNA that gives approximately 8% bead enrichment.

Note: Always use the same diluted sample that was quantified by PicoGreen as your template for emPCR amplification.

Option 2: Full Sequencing Titration Assay

1. Perform emulsion titration as described as above (Option 1).
2. Follow the instructions in the *GS FLX Titanium General Library Preparation Method Manual* beginning with section 3.10.3.2. Complete the full sequencing titration assay.

For large scale sequencing, use the library amount that gives the largest number of total passed filtering reads.

Step. 3. Where to Go From Here

You are now ready to proceed with emPCR amplification and 454 sequencing. Refer to the *GS FLX Titanium emPCR Method Manual* and the *GS FLX Titanium Sequencing Method Manual* for details.

Appendix A. Sequence Capture with Multi-Array Sets

This appendix describes issues pertaining to the use of multi-array sets for Sequence Capture of DNA samples. Suggested protocol modifications for using multi-array sets are detailed here and throughout the user's guide where applicable. Read this Appendix thoroughly before proceeding.

Considerations

- Some very large Sequence Capture targets (e.g. chromosome regions greater than 30Mb) may be divided among multiple arrays to provide sufficient coverage by oligonucleotide capture probes.
- Sequence Capture experiments using a multi-array set require proportionally more sstDNA library than experiments using a single array. Increase the number of library construction.
- As with any large-scale molecular biology experiment, including experiments using multi-array sets, we recommend that you generate extra library at the beginning of your experiment (Chapter 2). This helps ensure that sufficient material will be available for hybridization to multi-array sets if there are low yields during library preparation, or if there is a need to repeat hybridization to one array in a set without repeating the full set.
- When performing a Sequence Capture experiment using a multi-array set, we recommend you process your DNA sample as multiple independent 3-5µg preparations in parallel.
- To minimize variation in enrichment level among the different arrays of a multi-array set, it may be helpful to ensure the library material is as uniform as possible prior to the hybridizations. This *optional* step can be accomplished by pooling multiple library preparations, mixing them, and splitting them again immediately prior to hybridization (see “Instructions for Pooling Library Preparations for Hybridization to Multi-Array Sets” below).

Note: Pooling as a means to increase uniformity prior to hybridization should only be done using library preparations generated from the same original genomic DNA sample.

Instructions for Pooling Library Preparations for Hybridization to Multi-Array Sets

Note: If you are processing multiple DNA preparations in parallel from the same original sample for hybridization to a multi-array set, and have decided to pool and mix your multiple library preparations to increase their uniformity prior to hybridization, follow the protocol provided below:

1. After having confirmed that each library preparation was successful by Bioanalyzer analysis (Chapter 2, Step 2), pool the libraries into one 1.5ml tube and mix by pipetting up and down.
2. Measure and record the concentration of the pooled library.
3. Perform pre-capture LM-PCR (Chapter 3). Scale up the number of PCR reactions.
4. Pool the purified LM-PCR products that were generated in Chapter 3 into a single 1.5ml tube (these correspond to the library samples that were pooled earlier in this Appendix).
5. Calculate the total mass available for hybridization of each sample (concentration x total volume).

Important: You should have enough library to hybridize at least 3 μ g to each array in your multi-array set. If you have less than 3 μ g of library for each array, contact Roche Microarray Technical Support for additional guidance.

6. Store any remaining library at -20°C. This library could be used to repeat a hybridization to one of the arrays in the multi-array set.

Note: Save a sufficient amount of purified LM-PCR product for use in qPCR analysis (i.e. “pre-captured LM-PCR products,” Chapter 8).

7. Proceed to sample hybridization of the amplified, linker-ligated library (Chapter 3).

Appendix B. Limited Warranty

ROCHE NIMBLEGEN, INC. NIMBLEGEN ARRAYS

1. Limited Warranty

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