



# Instructions For Use

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## OmnISTR™ MPS Library Normalization Kit

qPCR and calculator

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**NimaGen.**

Innovators in  
DNA Sequencing  
Technologies



## Product and Company Information

### OmniSTR™ MPS Library Normalization Kit



LNQ-ASTR96

Research Use Only



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## Symbols Used on Product Labels and in Instruction For Use

Symbol	Description
	Manufacturer
	Use-by date
	Lot number
	Reference number
	Temperature limit for storage
	Contains sufficient for <n> tests
	GS1 DataMatrix, containing information about the product

## Product Description

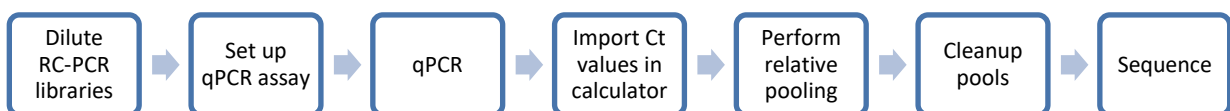
One of the challenges of STR profiling in forensic MPS is the diversity of sample quantity and quality, especially in casework and ancient DNA profiling. This results often in a high spread of signal strengths (CE) or read depth (MPS) within one set of samples. In case of MPS this can result in samples that generate allele read depths below analysis threshold, as well as samples that generate an excessive amount of reads, limiting the capacity of the flow cell and thus increasing costs. Therefore, a sample-to-sample normalisation is highly desirable in sample sets that are not proportionate regarding quantity and quality.

One strategy for this is to use a pooling strategy, based on predicted read depth using known DNA concentration and presumed quality. The drawback of this method is that estimations based on input do not always result in the expected library quantity. Another method often used, is to purify all individual libraries, followed by qPCR quantification. Disadvantages of this method are the high costs and hands-on time.

Introducing the innovative OmniSTR™ MPS Library Normalization Kit, based on a direct and easy qPCR in combination with an advanced calculator, NimaGen is further simplifying and improving MPS for forensics, by providing a simple, robust and easy workflow, resulting in optimized sample-to-sample read depth normalization. The method provides a complete detailed step-by-step pipetting scheme, generating best in class data using a wide range of quantity and/or quality of input material.

In contrast with qPCR based quantification methods for illumina® libraries using universal P5 and P7 adapter primers, this method is based on OmniSTR™ target specific, nested primer sets for qPCR. This results in highly specific relative quantification of a wide range of quantity and quality input samples.

## Schematic Workflow



## Reverse Complement PCR Kit Contents

NimaGen Part# LNQ-ASTR96 (store at -20 °C; protect from light)	Contents	
OmniSTR™ qPCR Primer Set (REF: RQP-ASTR96)	1x Tube (120 µL)	●
2x qPCR Master Mix (REF: MMRQP096)	1x Tube (1150 µL)	●

## Required Materials, Not Included

Description	Vendor
Samples created with the IDseek® OmniSTR™ kit	NimaGen
Real-Time qPCR Instrument, calibrated for SYBR® Green detection	Multiple Vendors
Adjustable Pipette Set (P10, P20, P100, P200, P1000)	Multiple Vendors
Optical 96-well qPCR plate, with seals or caps, compatible with the instrument used	Multiple Vendors
Standard 96-well PCR plate and seals/caps for making pre-dilutions	Multiple Vendors
Plate Spinner or centrifuge for 96-well PCR plates	Multiple Vendors
Molecular grade H <sub>2</sub> O	Multiple Vendors
Qubit Fluorometer incl. dsDNA High Sensitivity consumables	Thermo Fisher Scientific
TapeStation, Bioanalyzer Instrument, incl. consumables.	Agilent
NaOH Solution (2 N), MPS grade	Multiple Vendors
Tris-HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Illumina MPS Sequencing Instrument (MiSeq®/MiSeq FGx®)	illumina®/ Verogen®
Illumina MiSeq® Reagent kit v3 (600-cycle) or Verogen MiSeq FGx® Reagent Kit or Verogen MiSeq FGx® Reagent Micro Kit	illumina®/ Verogen®

## General Precautions


Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice when handling both the reagents supplied in this kit and other reagents required.

Use a Post-PCR environment for setting up the qPCR, sample pooling, purification and library quantification.

All reagents need to be thawed and centrifuged before use. Make sure to mix reagents and reactions properly.

## Protocol

### 1. Real-Time PCR Thermal Cycling Program

Step	Temp	Duration	Cycles
Initial denaturation	95 °C	45 seconds	1 x
Cycling	95 °C	7 seconds	30 x
	60 °C	5 seconds	
	68 °C	25 seconds* 	

#### \*Data collection at the end of extension step

**Note:** Use a standard method generating Ct values for each well, based on SYBR® Green. Example: “Comparative Ct ( $\Delta\Delta Ct$ )”. Use ROX normalization, when using an Applied Biosystems instrument.

**Note:** Page 8 shows a screenshot of this cycling program in QuantStudio™ Instrument software.

## 2. Setting up the qPCR reactions

### 2.1 Thaw on ice:

- OmniSTR™ qPCR Primer Set (Black cap)
- 2x qPCR Master Mix (Green cap)

**Note:** Always ensure that the Master Mix is fully thawed and thoroughly mixed before use.

### 2.2. Take a universal, 96-well PCR plate and dispense 148 µL molecular grade H<sub>2</sub>O in the applicable wells.

### 2.3. Mix the amplified (IDX RC-PCR / OmniSTR™) plate or strips by short vortexing, followed by a quick spin. Remove the caps or seal and transfer 2 µL of each well to the corresponding well of the plate from step 2.2, to make a 75x dilution of all libraries. This can be easily done by a multichannel pipette. After pipetting, close both plates with a seal or caps.

### 2.4. Mix the plate with diluted libraries by short vortexing, followed by a quick spin.

### 2.5. Create a qPCR mix by combining and mixing, per sample:

- 10 µL of qPCR Master Mix (Green Cap),
- 1 µL of OmniSTR™ qPCR Primer Set (Black Cap)
- 7 µL of PCR grade H<sub>2</sub>O.

**Example:** 24 samples + 10% extra volume\*

- 264 µL qPCR Master Mix
- 26.4 µL OmniSTR™ qPCR Primer Set
- 184.8 µL PCR grade H<sub>2</sub>O

\*It is recommended to allow for a 10% excess when preparing the qPCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.

### 2.6. Take a new Real-Time PCR compatible (optical) 96-well plate and dispense 18 µL of the qPCR mix from step 2.5 to each well of the plate.

### 2.7. Add to each well 2 µL of the 75x diluted RC-PCR sample (from step 2.3).

### 2.8. Close the qPCR plate using an optical seal or optical caps.

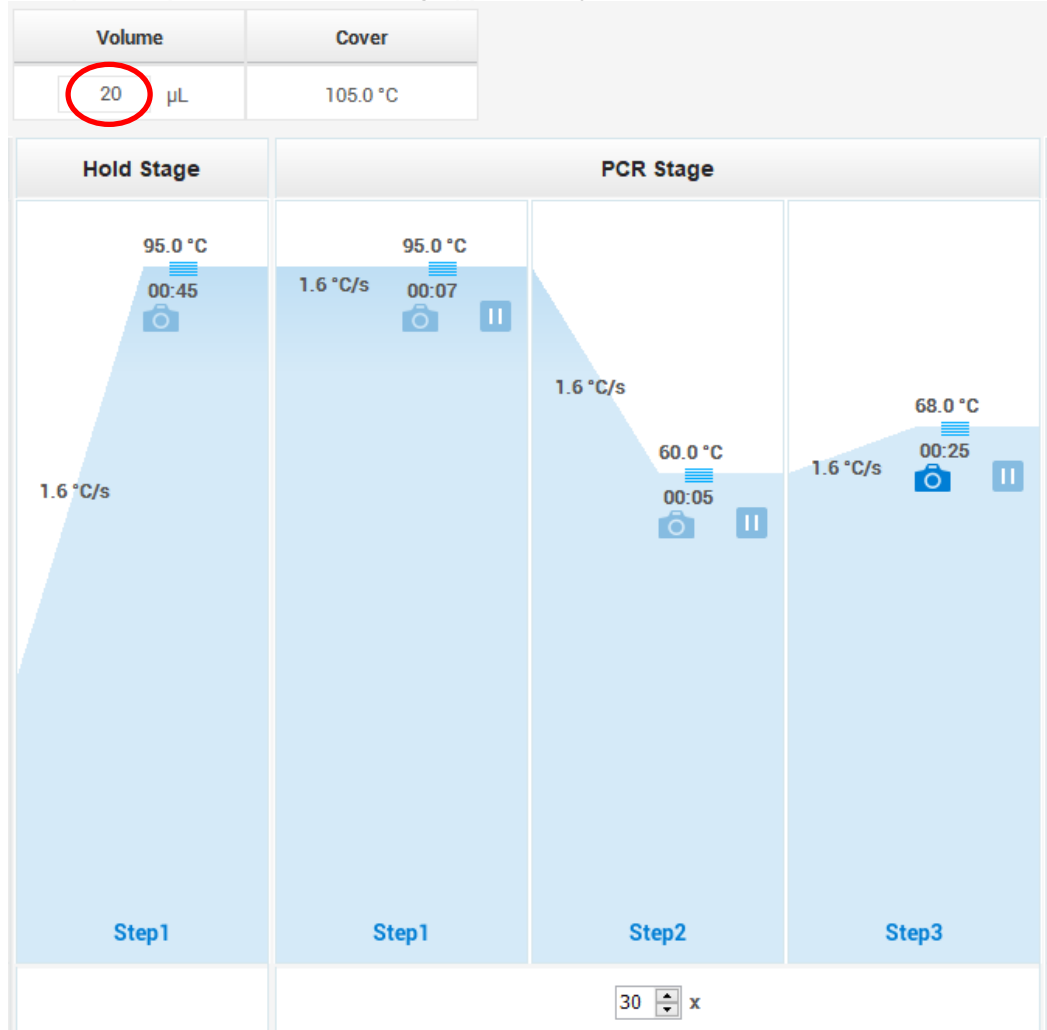
### 2.9. Place the samples in the qPCR instrument, and start the qPCR program.

### 2.10. Once the run is completed, export the results file and continue to chapter 3.

**Example of Experimental Properties using Applied Biosystems QuantStudio™ instruments:**

Properties	Method	Plate	Run	Results	Export
<b>Experiment Properties</b>					
Name	Example Quantification OmniSTR				
Barcode	Barcode - optional				
User name	User name - optional				
Instrument type	QuantStudio™ 1 System				
Block type	96-Well 0.2-mL Block				
Experiment type	Comparative Ct (ΔΔCt)				
Chemistry	SYBR® Green Reagents				
Run mode	Standard				

**Example of Experiment Method using Applied Biosystems QuantStudio™ instruments:**





### 3. IDseek® OmniSTR™ Global Library qPCR Normalization Calculator, Tab 1 (“Enter Ct Values”)

- 3.1 Open the IDseek® OmniSTR™ Global Library qPCR Normalization Calculator.
- 3.2 Open the results files from the qPCR run in Excel, including the standard Ct values per well. Make sure to sort by column (A01, B01, C01 etc.) or by row (A01, A02, A03 etc.).
- 3.3 Copy the column with the Ct Values and paste it in the first tab of the calculator at the column “Ct value”. Check that well positions are in line with the original RC-PCR plate.

Well pos.	Targeted read depth	Suggested read depth	Ct value	Sample/NTC	Optional: Sample Name	Pool#
A01	30000	NA	0	Sample		NA
B01	30000	NA	0	Sample		NA
C01	30000	NA	0	Sample		NA
D01	30000	NA	0	Sample		NA
E01	30000	NA	0	Sample		NA
F01	30000	NA	0	Sample		NA
G01	30000	NA	0	Sample		NA
H01	30000	NA	0	Sample		NA
A02	30000	NA	0	Sample		NA
B02	30000	NA	0	Sample		NA
C02	30000	NA	0	Sample		NA

3.4 For well positions containing No Template Controls (negative controls): change the column “Sample/NTC” to “NTC”.

**Important:** All empty well positions MUST have the value: 30000 in the column “Targeted read depth”.

- 3.5 The automated algorithm now assigned all individual libraries from the RC-PCR plate to one of the following pools:
  - o 1 (blue, high concentration)
  - o 2 (green, medium concentration)
  - o 3 (yellow, low concentration)
  - o 4 (orange, very low concentration)

3.6 The calculator suggests the needed read depth for each individual sample in column 4 “Suggested read depth”. It is recommended to adhere to this suggestion. This can be done by clicking the “Transfer suggested read depth” button. If the values in column 2 and column 4 are identical, there will be a “√” in column 3.

In certain cases, there are reasons to increase the targeted read depth, and overrule the suggested read depth. Those reasons can be:

- o (Potential) mixed samples, especially with a high ratio between minor and major contributor(s).
- o Important casework samples that need extra insurance of enough read depth. This is especially important with low quality samples, where the interlocus balance can be affected.

**Important:** Changing read depth from the standard 30000 to a higher value, can cause the sample well to move to another pool. This is caused by the background calculations based on volumes needed.

Example of Tab 1 ("Enter Ct Values"):

IDseek® OmniSTR™ Global Library qPCR Normalization Calculator							
Well pos.	Targeted read depth	↔	Suggested read depth	Ct value	Sample/NTC	Optional: Sample Name	Pool#
A01	30000	✓	30000	9,39921093	Sample	1ngM1	1
B01	30000	✓	30000	9,634009361	Sample	1ngM2	1
C01	30000	✓	30000	11,29136372	Sample	250pgM1	1
D01	30000	✓	30000	12,32950497	Sample	125pgM1	2
E01	30000	✓	30000	13,95950508	Sample	62.5pgM1	2
F01	100000	✓	100000	15,03899384	Sample	31.25pgM1	3
G01	300000	✓	300000	16,51139641	Sample	15.625pgM1	4
H01	30000	✓	30000	9,338350296	Sample	Saliva-direct1	1
A02	30000	✓	30000	9,365641594	Sample	1ngM3	1
B02	30000	✓	30000	9,012501717	Sample	1ngM4	1
C02	30000	✓	30000	10,77439308	Sample	250pgM2	1
D02	30000	✓	30000	12,08170219	Sample	125pgM2	2
E02	300000	✗	30000	13,29296112	Sample	Mixed 62.5pgM2/M1	3
F02	100000	✓	100000	14,8880558	Sample	31.25pgM2	3
G02	300000	✓	300000	16,98287678	Sample	15.625pgM2	4
H02	30000	✓	30000	9,35675478	Sample	Saliva-direct2	1
A03	30000	✓	30000	9,160629272	Sample	1ngF1	1
B03	30000	✓	30000	8,717593193	Sample	1ngF2	1
C03	30000	✓	30000	11,01090164	Sample	250pgF1	1
D03	30000	✓	30000	12,08310089	Sample	125pgF1	2
E03	30000	✓	30000	12,98008633	Sample	62.5pgF1	2
F03	100000	✓	100000	14,66183281	Sample	31.25pgF1	3
G03	300000	✓	300000	16,80181599	Sample	15.625pgF1	4
H03	30000	✓	30000	10,01591015	Sample	Saliva-direct3	1
A04	30000	✓	30000	8,956582546	Sample	1ngF3	1
B04	30000	✓	30000	10,87173653	Sample	1ngF4	1
C04	30000	✓	30000	10,88969517	Sample	250pgF2	1
D04	30000	✓	30000	12,10948772	Sample	125pgF2	2
E04	30000	✓	30000	12,63413239	Sample	62.5pgF2	2
F04	100000	✓	100000	14,68572044	Sample	31.25pgF2	3
G04	300000	✓	300000	16,62050247	Sample	15.625pgF2	4
H04	30000	✓	30000	9,544948578	Sample	Saliva-direct4	1
A05	30000	✓	30000	26,65040588	NTC	NTC1	1
B05	30000	✓	30000	26,14789009	NTC	NTC2	1
C05	30000	NA	0		Sample		NA
D05	30000	NA	0		Sample		NA
E05	30000	NA	0		Sample		NA

- Column 1:** **Well position.** Check for correctness according to original RC-PCR plate.
- Column 2:** **Targeted Read depth.** Manually assignable. As a guidance, use the suggested read depth from column 4. NOTE: The value in this column MUST be 30000 for empty wells.
- Column 3:** **✗.** Indicates if there is a difference between targeted read depth and suggested read depth. In the example, for well E02, the targeted read depth had been assigned higher than the suggested read depth (see column 4), because the original sample was a mix.
- Column 4:** **Suggested read depth.** Based on  $\Delta\Delta Ct$ . Important note: to correctly calculate this, there must be at least one sample in the plate with a concentration of  $\geq 1$  ng and good quality (positive control). NimaGen recommends to use 2800M as a positive control sample.
- Column 5:** **Ct value.** Paste here the calculated Ct values from the qPCR. The field with the lowest Ct (highest concentration) will be displayed in dark green. Check if this well position contains (one of the) highest concentration DNA input.
- Column 6:** **Sample/NTC.** Default all lines are "sample". Change this to NTC for wells containing No Template Controls. NTC samples will always have a suggested read depth of 30000. Ct values of NTC generally are  $>20$ . NOTE: NTC will still generate a certain Ct value in this assay. The analysis of NTC controls will take place after analysis of the sequencing data.
- Column 7:** **Sample Name.** Optional: fill in sample name and /or concentration.
- Column 8:** **Pool#.** The pool to where the sample is assigned, including color code.

## 4. Create library pools, Tab 2 (“Pooling Scheme”)

4.1. After finishing the calculator Tab 1 (“Enter Ct Values”), click on the Tab 2 (“Pooling Scheme”) of the calculator. This tab displays a pipetting scheme for creating 1, 2, 3 or 4 pools from the RC-PCR plate. In the example below, take 4 new 1.5 mL tubes with flip cap and label them with resp. P1, P2, P3 and P4. Each position in the scheme (see below) displays the allocated Pool for each sample, as well as the volume (in  $\mu\text{L}$ ). Wells with “N” are empty.

**Example:** Pipette 4.44  $\mu\text{L}$  of the RC-PCR product from position A1 to the tube, labelled with “P1”.

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1: 4,44	P1: 4,34	P1: 3,78	P1: 3,29	P1: 11,2	N	N	N	N	N	N	N
B	P1: 5,21	P1: 3,42	P1: 2,8	P1: 12,04	P1: 11,2	N	N	N	N	N	N	N
C	P1: 16	P1: 11,27	P1: 13,23	P1: 12,19	N	N	N	N	N	N	N	N
D	P2: 5,3	P2: 4,48	P2: 4,49	P2: 4,57	N	N	N	N	N	N	N	N
E	P2: 16	P3: 14,35	P2: 8,24	P2: 6,52	N	N	N	N	N	N	N	N
F	P3: 16	P3: 14,45	P3: 12,39	P3: 12,6	N	N	N	N	N	N	N	N
G	P4: 16	P4: 16	P4: 16	P4: 16	N	N	N	N	N	N	N	N
H	P1: 4,26	P1: 4,32	P1: 6,74	P1: 4,9	N	N	N	N	N	N	N	N

**NOTE:** The pipetting of pools can be easily automated by a liquid handler, able to pipette volumes from 1.5 – 16  $\mu\text{L}$ . For this purpose, use Tab 5 (“Export for Liquid Handler”).

## 5. Purification using the pipetting scheme, Tab 3 (“Beads Cleanup”)

The purification involves one-sided size selection using magnetic beads, minimizing the number of reads lost to residual primers and primer-dimers.

5.1. Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to **room temperature**.

5.2. Follow the Bead Cleanup Procedure as described in Tab 3 (“Beads Cleanup”).

**Notes:** Additional remarks for Bead Cleanup procedure:

- a. It is of high importance to bring the magnetic bead solution to room temperature before starting the purification. In case of large volumes, it is advisable to use smaller aliquots.
- b. Steps 2 and 21: After adding the beads solution pipette up and down to mix immediately, or short vortex. Optionally short spin to ensure full volume is at the bottom of the tube.
- c. Always use freshly prepared 75% Ethanol.
- d. Make sure to not disturb the bead pellet, while pipetting to a tube on magnet.

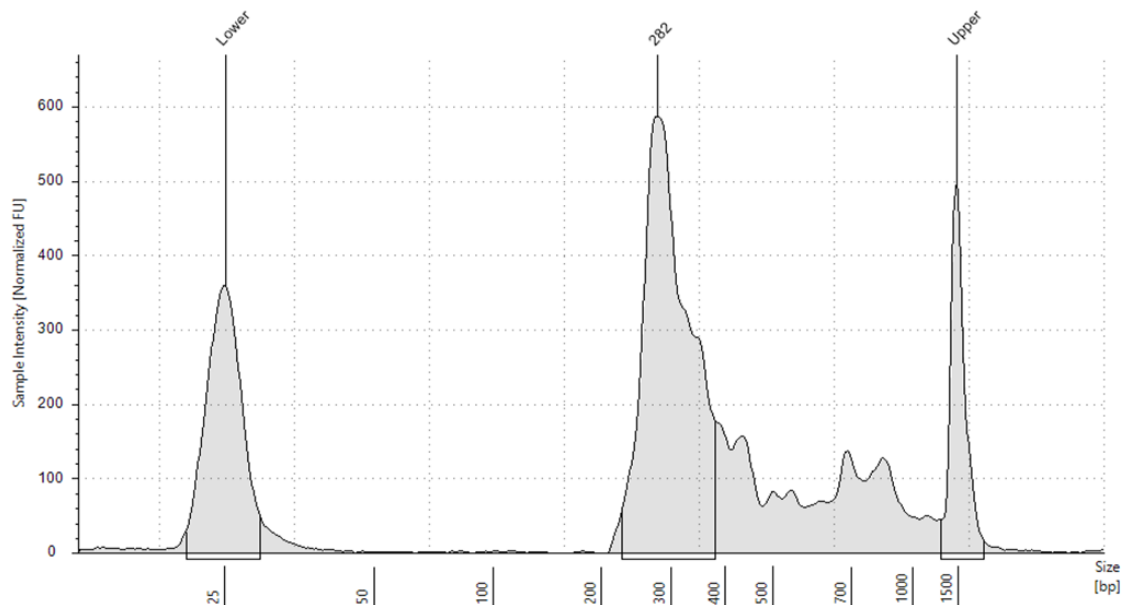
## 6. Prepare for sequencing, Tab 4 ("Combine Pools and Final Steps")

- 6.1. Determine the final concentration of the library or libraries by a Qubit (HS) measurement:
- Bring the Qubit reagents to room temperature.
  - Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
  - Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer for each sample/ standard. It is recommended to allow for >10% excess when preparing the working solution to correct for any pipetting loss.
  - For the standards: mix 190  $\mu$ L of the working solution with 10  $\mu$ L of the standard.
  - For the samples: mix 180-199  $\mu$ L of the working solution with 1-20  $\mu$ L sample (total 200  $\mu$ L).
  - Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
  - Measure the standards and the samples using the "dsDNA High Sensitivity" settings making sure to select the correct sample volume used in step e..

**Note:** The quantification method may be adapted to an in-house available and/or preferred method, such as qPCR. When quantifying using TapeStation or Bioanalyzer, we recommend to reduce the initial loading concentration of the library on the sequencer to avoid overclustering.

- 6.2. **Optional but recommended:** Perform a qualitative verification of the library on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the pool. E.g. for TapeStation High Sensitivity kit, dilute to ~2 ng/ $\mu$ L.

*Example of a clean library on TapeStation:*



- 6.3. Prepare a sample sheet for the MiSeq® or MiSeq FGx® in RUO mode.
- 6.4. Open Tab 4 ("Combine Pools and Final Steps") and prepare the final sequencing library following the pipetting guidelines.

**Notes:** Additional remarks for Sequencing preparation:

Step 2: Combine libraries in one tube:		
μL of 5x Diluted POOL 1:	3.4 μL	of 5x Diluted Pool 1
μL of POOL 2:	1.4 μL	
μL of POOL 3:	1.5 μL	
μL of POOL 4:	13.7 μL	WARNING
μL EBT Buffer:	0.0 μL	
<b>TOTAL OF COMBINED POOL:</b>	<b>20.0 μL</b>	<b>2 nM</b>

- In case the red box "of 5x Diluted Pool 1" pops up, Pool 1 needs to be diluted 5x before adding the advised volume to the Combined Pool. In case the red box "of 20x Diluted Pool 1" pops up, Pool 1 needs to be diluted 20x before adding the advised volume to the Combined Pool.
- In case the "WARNING" signal pops up, the volume required is lower than the obtained volume for the pool after purification, Qubit and TapeStation. In this case we advise to adjust the final pool volume, for example:  
Lower the final volume with 25%, to "TOTAL OF COMBINED POOL": 15 μL (2nM). This leads to combining the following volumes:
 

μL of 5x Diluted POOL 1:	2.55	μL
μL of POOL 2:	1.05	μL
μL of POOL 3:	1.13	μL
μL of POOL 4:	10.28	μL
<b>TOTAL OF COMBINED POOL:</b>	<b>15</b>	<b>μL 2nM</b>

In case of doubt reach out to your NimaGen contact person for assistance.
- A spike-in of 25% PhiX is required for QC purposes and to act as a safeguard against colour-space issues.
- The loading concentration can be adapted in the "Loading Concentration (fill in)" box. We advise to start with a lower loading concentration for the initial sequence run and adjust in subsequent runs if needed. This avoids overclustering and potentially failure of the run. For the MiSeq® a concentration of 8 pM is recommended.

## Data Analysis

Open the results file from your qPCR run via Excel. Copy the Ct values from your Excel in the IDseek® OmniSTR™ Library qPCR Normalization Calculator and follow the procedure described from chapter 3.

The IDseek® OmniSTR™ kit does not come with predetermined data analysis software, instead the user is given full freedom to implement the analysis tools which best suits their needs. Several options already include the OmniSTR™ kit as a preset library including commercial software solutions such as MixtureAce™ (NicheVision®), or open-source software such as FDSTools (Netherlands Forensic Institute), STRait Razor online and STRait Razor v3 (The University of North Texas Health Science Centre).

Due to its size, larger alleles of SE33 cannot be sequenced fully from one side and could require merging of the two individual reads into a single long fragment for downstream analysis. The read 1 and 2 fastq files can be merged using a tool such as 'Fast Length Adjustment of Short reads' (FLASH) (Centre for Computational Biology, John Hopkins University).

## Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at [techsupport@nimagen.com](mailto:techsupport@nimagen.com).

## Revision History

Section	Summary of changes	Version	Date
All	Version 1	1	2024-09-02



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