Instructions For Use

Version: 1.1 *Ref: IFU_LNQ-YSTR* *Revision date: 2025-10-08*

mYSTR™ MPS Library Normalization Kit

qPCR and calculator

NimaGen.

Innovators in DNA Sequencing Technologies

Product and Company Information

mYSTR™ MPS Library Normalization Kit

REF LNQ-YSTR96

Research Use Only

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Symbols Used on Product Labels and in Instruction For Use

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

Product Description

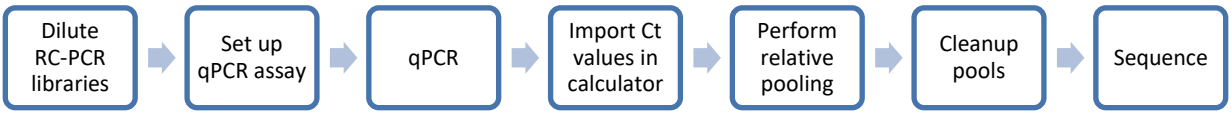
One of the challenges of STR profiling in forensic MPS is the diversity of sample quantity and quality, especially in casework and ancient DNA profiling. This results often in a high spread of signal strengths (CE) or read depth (MPS) within one set of samples. In case of MPS this can result in samples 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 mYSTR™ 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 mYSTR™ 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-YSTR96 (store at -20 °C; protect from light)	Contents
mYSTR™ qPCR Primer Set (REF: RQP-YSTR96)	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® mYSTR™ 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_2O	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 (ΔΔCt)". 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:
- mYSTR™ 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_2O in the applicable wells.

2.3. Mix the amplified (IDX RC-PCR / mYSTR™) 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 mYSTR™ qPCR Primer Set (Black Cap)
- 7 µL of PCR grade H_2O

Example: 24 samples + 10% extra volume*
- 264 µL qPCR Master Mix
- 26.4 µL mYSTR™ qPCR Primer Set
- 184.8 µL PCR grade H_2O

*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.

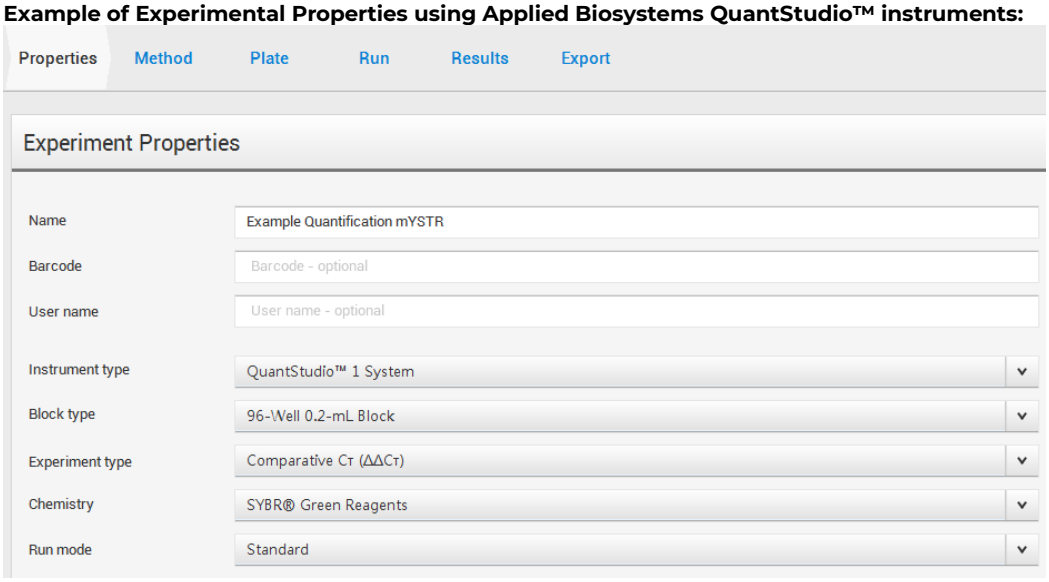
2.7. Add to each well 2 µL of the 75x diluted RC-PCR sample (from step 2.3).

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

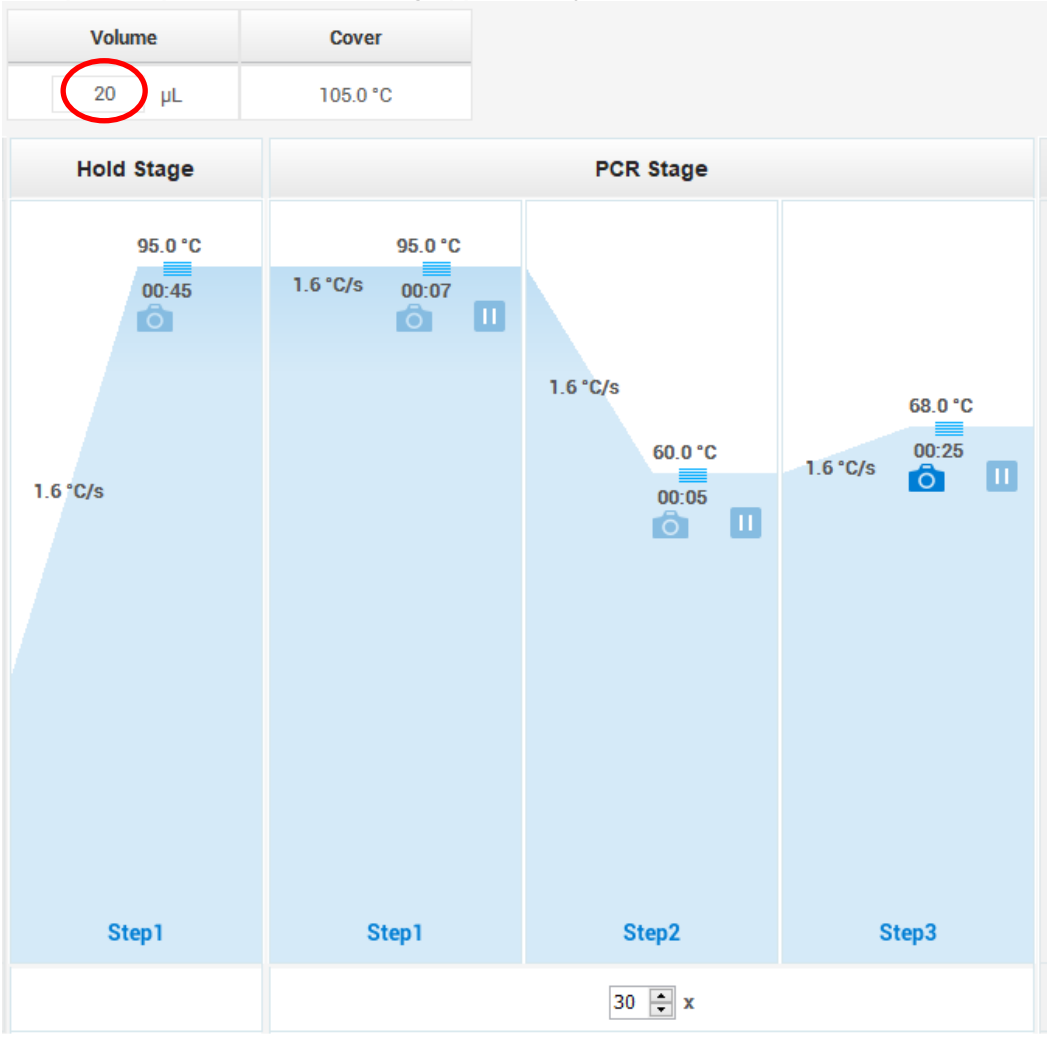
2.9. Place the samples in the qPCR instrument, and start the qPCR program.

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:



Example of Experiment Method using Applied Biosystems QuantStudio™ instruments:

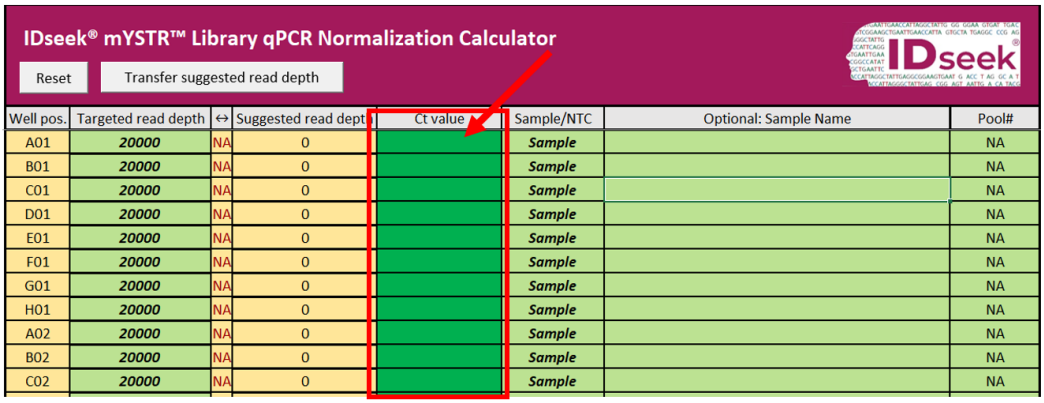


3. IDseek® mYSTR™ Library qPCR Normalization Calculator, Tab 1 (''Enter Ct Values'')

3.1 Open the IDseek® mYSTR™ 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.



IDseek® mYSTR™ Library qPCR Normalization Calculator

Reset | Transfer suggested read depth

Well pos.	Targeted read depth	↔	Suggested read dept	Ct value	Sample/NTC	Optional: Sample Name	Pool#
A01	20000	NA	0		Sample		NA
B01	20000	NA	0		Sample		NA
C01	20000	NA	0		Sample		NA
D01	20000	NA	0		Sample		NA
E01	20000	NA	0		Sample		NA
F01	20000	NA	0		Sample		NA
G01	20000	NA	0		Sample		NA
H01	20000	NA	0		Sample		NA
A02	20000	NA	0		Sample		NA
B02	20000	NA	0		Sample		NA
C02	20000	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: 20000 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:

- 1 (blue, high concentration)
- 2 (green, medium concentration)
- 3 (yellow, low concentration)
- 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:

- (Potential) mixed samples, especially with a high ratio between minor and major contributor(s).
- 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 20000 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® mYSTR™ Library qPCR Normalization Calculator

Reset | Transfer suggested read depth

Well pos.	Targeted read depth	↔	Suggested read depth	Ct value	Sample/NTC	Optional: Sample Name	Pool#
A01	20000	✓	20000	8.475067139	Sample	1ngM	1
B01	20000	✓	20000	9.356801987	Sample	500pgM	1
C01	20000	✓	20000	10.19921398	Sample	250pgM	1
D01	20000	✓	20000	11.24355698	Sample	125pgM	1
E01	20000	✓	20000	12.97734261	Sample	62.5pgM	2
F01	20000	✓	20000	13.3159256	Sample	31.25pgM	2
G01	60000	✓	60000	15.45256233	Sample	15.625pgM	3
H01	200000	✓	200000	16.25539379	Sample	7.8125pgM	4
A02	20000	✓	20000	8.321195602	Sample	1ngF	1
B02	20000	✓	20000	9.100521088	Sample	500pgF	1
C02	20000	✓	20000	10.3926754	Sample	250pgF	1
D02	20000	✓	20000	11.18447304	Sample	125pgF	1
E02	20000	✓	20000	12.40611267	Sample	62.5pgF	2
F02	20000	✓	20000	13.64793015	Sample	31.25pgF	2
G02	60000	✓	60000	14.30874062	Sample	15.625pgF	3
H02	200000	✓	200000	15.84458618	Sample	7.8125pgF	4
A03	200000	≠	20000	13.29296112	Sample	Mixed 62.5pgM/F	3
B03	20000	✓	20000	11.48165985	Sample	Saliva-direct1	1
C03	20000	✓	20000	12.95883656	Sample	Saliva-direct2	2
D03	200000	✓	200000	16.16894436	Sample	Fingerprint1	4
E03	60000	✓	60000	15.66643238	Sample	Fingerprint2	3
F03	20000	✓	20000	10.66131878	Sample	Swab1	1
G03	20000	✓	20000	10.05105019	Sample	Swab2	1
H03	20000	✓	20000	25.65300941	NTC	NTC1	1
A04	20000	✓	20000	25.67226601	NTC	NTC2	1
B04	20000	NA	0		Sample		NA
C04	20000	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 20000 for empty wells.*

Column 3: **≠.** *Indicates there is a difference between Targeted read depth and Suggested read depth. In the example, for well A03, 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 ΔΔCt. Important note: to correctly calculate this, there must be at least one sample in the plate with a concentration of ≥1 ng and good quality (positive control). NimaGen recommends using 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 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 20000. 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 which 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 µL). Wells with “N” are empty.

Example: Pipette 2.09 µL 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: 2.09	P1: 1.88	P3: 10.4	P1: 7.52	N	N	N	N	N	N	N	N
B	P1: 3.79	P1: 3.19	P1: 16	N	N	N	N	N	N	N	N	N
C	P1: 6.71	P1: 7.65	P2: 10.03	N	N	N	N	N	N	N	N	N
D	P1: 13.62	P1: 13.08	P4: 16	N	N	N	N	N	N	N	N	N
E	P2: 10.16	P2: 6.9	P3: 16	N	N	N	N	N	N	N	N	N
F	P2: 12.78	P2: 16	P1: 9.18	N	N	N	N	N	N	N	N	N
G	P3: 13.84	P3: 6.38	P1: 6.07	N	N	N	N	N	N	N	N	N
H	P4: 16	P4: 16	P1: 7.52	N	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 µL. 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 the 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”)

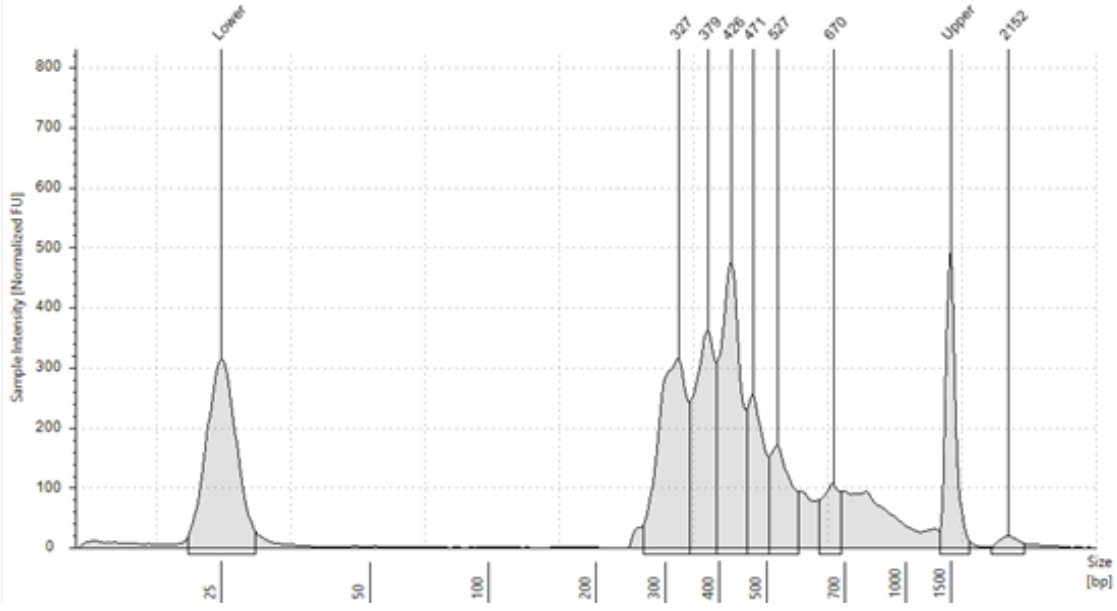
6.1. Determine the final concentration of the library or libraries by a Qubit (HS) measurement:

a. Bring the Qubit reagents to room temperature.
b. Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
c. 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.
d. For the standards: mix 190 µL of the working solution with 10 µL of the standard.
e. For the samples: mix 180-199 µL of the working solution with 1-20 µL sample (total 200 µL).
f. Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
g. 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/µL.

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	
TOTAL OF COMBINED POOL:	20.0 µL	2 nM

a. 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.

b. 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	
TOTAL OF COMBINED POOL:	15	µL	2nM

In case of doubt reach out to your NimaGen contact person for assistance.

c. A spike-in of 25% PhiX is required for QC purposes and to act as a safeguard against colour-space issues.

d. 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® mYSTR™ Library qPCR Normalization Calculator and follow the procedure described from chapter 3.

The IDseek® mYSTR™ 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 mYSTR™ 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).

Merging of the two individual reads into a single long fragment for downstream analysis is advised. 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), or by using the merging option in MixtureAce™ (NicheVision®).

Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at techsupport@nimagen.com.

Revision History

Section	Summary of changes	Version	Date
All	First Version.	1	2024-09-02
Ref	Ref changed from IFU-LNQ-YSTR96 to IFU_LNQ-YSTR.	1.1	2025-10-08

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NimaGen B.V.
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