



Title: WI, Nextera Flex for Enrichment SMB QC Assay

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Purpose	1
Scope	1
Responsibilities	2
References	2
Safety & General Precautions	2
Equipment/Materials	3
Equipment	3
Tested Materials	3
Consumables	4
Equipment Startup & Setup Procedure	5
Equipment Shutdown Procedure	6
Definitions	6
Work Instruction	7
Procedure	8
1 Library Tagmentation and Cleanup	8
2 Library PCR	11
3 PCR Clean Up and Pooling	13
4 Hybridization	17
5 Capture	18
6 Second PCR and Clean Up	21
7 Sequencing Preparation	23
8 Data Analysis and Reporting	26
Release History	34

Purpose

The objective of this Work Instruction (WI) is to provide a protocol for QC testing of Nextera Flex for Enrichment Streptavidin Magnetic Beads (SMB) tube parts.

Scope

This document applies to QC Reagent Test in San Diego, California.



Responsibilities

Role/Function	Responsibilities
Reagent Test	It is the responsibility of Reagent Test to perform Quality Control testing using the protocol found in this work instruction according to <i>Procedure Reagent Quality Control, 15029291</i> .

References

Title	Reference Number
Cipher 24 UDI Pre-Verification Report	1000000043872
Engineering Report, Titanium QC Full Functional Assay	1000000069709
SMB NFE QC Specification Setting Engineering Report	1000000103688
Procedure, Out of Specification Procedure	15026737
Procedure Reagent Quality Control	15029291
SampleSheet, SMB NFE	1000000095063
Titanium Verification Protocol IC Report	1000000063709
WI, Gowning and Personnel Practices in Reagent Quality Control Lab	15048526
WI, NextSeq Reagent Sequencing QC	15049213
WI, Quantification for Sample Prep	15024021

Safety & General Precautions

- Refer to 15048526, WI, Gowning and Personnel Practices in Reagent Quality Control Lab
- Safety glasses, lab coat, and gloves are mandatory.
- Safety data sheets (SDS's) are available on the Illumina website.



Equipment/Materials

For equipment that is not available for an instance of a test, a supervisor may approve the use of equivalent equipment as defined in document WI 15029291, Procedure, Reagent Quality Control.

Equipment

Equipment	Supplier
1000 µL pipette	General lab supplier
200 µL pipette	General lab supplier
20 µL pipette	General lab supplier
200 µL multi-channel pipette	General lab supplier
20 µL multi-channel pipette	General lab supplier
Vortex	General lab supplier
Microfuge	General lab supplier
Table top centrifuge	General lab supplier
Plate shaker (Bioshake iQ)	Qinstruments
Thermal Cycler (Bio-Rad C1000 recommended)	General lab supplier
Dyna Mag magnetic stand – Strip magnet for PCR plate	General lab supplier
Dyna Mag magnetic stand – Peg magnet for MIDI plate	General lab supplier
Ice Bucket	General lab supplier
Hybex Incubator	SciGene
Timer	General lab supplier

Tested Materials

Material Number	Description	Type
15039631	TC#-SMB, STRPTVDN MAGBDS, FIN RGT 1.2 mL	Enrichment
20033748	SO#-SMB, STRPTVDN MAGBDS, uHT	Enrichment
20029947	SO#-SMB, Streptavidin Magnetic Beads	Enrichment

Note: One Enrichment reagent may be tested at one time.



Title: WI, Nextera Flex for Enrichment SMB QC Assay

1000000103802, 00

Page 4 of 34

Consumables

For Illumina-manufactured reagents and general lab consumables that are not available for an instance of a test, a supervisor may approve the use of equivalent reagents and equivalent consumables as defined in document WI 15029291, Procedure, Reagent Quality Control.

Consumable	Material Number	Supplier	Storage Temperature
LP#-TB1, TAGMENTATION BUFFER, FIN REAG	20015171	Illumina	-25°C to -15°C
HORIZON HD730 gDNA	20029632 (Illumina MN)	Horizon	2°C to 8°C
Molecular Grade Water	N/A	General lab supplier	15°C to 30°C
EBLTS	20024592, 20024594, or 20033740	Illumina	2°C to 8°C
TC#-ST2,STOP TAGMENT BUFFER 2,FIN REAG	15070420 (or equivalent)	Illumina	15°C to 30°C
LP#-TWB, TAGMENT WASH BUFFER, FIN REAG	20015079 (or equivalent)	Illumina	2°C to 8°C
LP#- RSB,RESUSPENSION BUFFER	15052041 (or equivalent)	Illumina	15°C to 30°C
LP#-EPM, ENHANCED PCR MIX, FR.20 ml	15071202 (or 20026390)	Illumina	-25°C to -15°C
96 UDI PCR INDX SetA	20026121	Illumina	-25°C to -15°C
TC#-SPB,SMP PURIF BEADS	20024094 (or equivalent)	Illumina	2°C to 8°C
Ethanol	N/A	General lab supplier	15°C to 30°C
Qubit dsDNA BR Assay Kit	Q32850 (or equivalent)	Thermo Fisher	2°C to 8°C
NHB1 or NHB2	20024596, 20029945, 20031771, or 20033755	Illumina	-25°C to -15°C
TS#-ONE, TruSight™ ONE OLIGOS,Fin Reag	15046658	Illumina	-25°C to -15°C
TC#-EHB2, Enrich Hyb Buffer 2	15070416 (or equivalent)	Illumina	2°C to 8°C
TC#-EEW. Enhanced Enrich Wash BFFR, FIN RGТ	15065792 (or equivalent)	Illumina	-25°C to -15°C



Title: WI, Nextera Flex for Enrichment SMB QC Assay
1000000103802, 00
Page 5 of 34

Consumable	Material Number	Supplier	Storage Temperature
TC#-ET2.FINISH REAG,ELUTE TARGET BFFR 2	15013008 (or equivalent)	Illumina	2°C to 8°C
TC#-EE1, Enrich ELUTION BFFR 1,FIN REAG	15037034 (or equivalent)	Illumina	-25°C to -15°C
GA#-HP3,FINISHED REAGENT	11324596 (or equivalent)	Illumina	-25°C to -15°C
LP#-PPC, PCR PRMR COCKTAIL, 0.32ML, FIN REA	15031748	Illumina	-25°C to -15°C
GA#-HT1,FINISHED REAGENT	11324502 (or equivalent)	Illumina	-25°C to -15°C
NSQ® 500 hi- Output RGT CART v2 (300 CYS)	15057929 (or equivalent)	Illumina	-25°C to -15°C
NextSeq® 500/550 Buffer Cartridge v2	15057941 (or equivalent)	Illumina	15°C to 30°C
20022408 NextSeq® High Output Flow Cell v2.5	20022408 (or equivalent)	Illumina	2°C to 8°C
96 well Hard Shell PCR Plate	N/A	General lab supplier	N/A
96 well MIDI plate	N/A	General lab supplier	N/A
1.5 mL Eppendorf tubes (or equivalent)	N/A	General lab supplier	N/A
20 µL pipette tips	N/A	General lab supplier	N/A
200 µL pipette tips	N/A	General lab supplier	N/A
1000 µL pipette tips	N/A	General lab supplier	N/A
Microseal 'A' (or equivalent)	MSA5001	Bio-Rad	N/A
Microseal 'B' (or equivalent)	MSB1001	Bio-Rad	N/A
PCR 8-tube strip	N/A	General lab supplier	N/A

Equipment Startup & Setup Procedure

N/A



Equipment Shutdown Procedure

N/A

Definitions

Term	Definition
WI	Work Instruction
QC	Quality Control
PCR	Polymerase Chain Reaction

Work Instruction

Workflow			
	Step	Hands on Time	Total Time
Library Preparation	Tagmentation and Clean Up	50 Minutes	60 Minutes
	PCR and Pooling	45 Minutes	75 Minutes
Enrichment	Hybridization	20 Minutes	145 Minutes
	Capture	40 Minutes	85 Minutes
	Second PCR and Cleanup	40 Minutes	75 Minutes
Sequencing	Sequencing	30 Minutes	30 Hours
Data Analysis	Automatic Data Analysis		6 Hours

Note: This workflow is performed in Post-PCR lab environment

Procedure

1 Library Tagmentation and Cleanup

- Hands on time: approximately 50 minutes
- Total time: approximately 60 minutes

1.1 Reagent Preparation

Reagent	Preparation
5X Tagmentation Buffer (TB1)	<ul style="list-style-type: none">• Thaw on ice• Vortex and spin
HD730 (DNA Sample)	<ul style="list-style-type: none">• Thaw on ice• Vortex and spin
Molecular Grade Water	<ul style="list-style-type: none">• Make an aliquot for use (at least 300 µL)• May be kept at room temperature
eBLT	<ul style="list-style-type: none">• Tube must be stored upright• Bring beads to room temperature• Pulse vortex just prior to using, ensure reagent is homogenous.• Centrifugation is not recommended, if tube must be centrifuged, re-suspend beads using pipette mixing.• Tube is single use
ST2	<ul style="list-style-type: none">• Vortex and spin• Check carefully for precipitates. If present, vortex until gone. If precipitates remain, a new tube may be used.• ST2 should always be stored at room temperature
TWB	<ul style="list-style-type: none">• Bring to room temperature• Invert to mix, minimize foaming• Check carefully for precipitates
RSB	N/A, may be kept at room temperature

1.2 Procedure Steps

1. Label a new Hard-shell PCR plate with **TiLP-1**, initials, date, and plate ID.

2. Prepare Tagmentation Master Mix in a 1.5 mL Eppendorf tube for 12 samples in the order listed, do not let beads settle:

Reagent	Volume per sample (μL)	Volume (12 Samples)* (μL)
Molecular Grade Water	20	276
RSB	9	124.2**
HD730 (DNA Sample) (50 ng/ μl)	1	13.8**
TB1	10	138
Small Insert Library Prep beads (eBLT)	10	138
Total	50	690

* Includes 15% overage

**Volumes are for starting concentration of 50 ng/ μl , if a different starting concentration is used, adjust accordingly. Final DNA input is 50 ng per sample, or 690 ng total in master mix with overage as outlined above.

3. Pulse vortex (no longer than 3 seconds) and quick spin (about 1 second, just enough to remove reaction from lid) just prior to use. Do not over-spin Tagmentation Master Mix, this will lead to beads settling. If beads settle, pipette mix until homogenous. Tagmentation Master Mix is stable at room temperature.
4. Using a single-channel pipette, add 50 μL of Tagmentation Master Mix to 12 wells each of a Hard-Shell PCR plate. See **Figure 1**.

Figure 1: TiLP-1 Plate Layout (Recommended)

	1	2	3	...	12
A	Replicate-1		Replicate-7		
B	Replicate-2		Replicate-8		
C	Replicate-3		Replicate-9		
D	Replicate-4		Replicate-10		
E	Replicate-5		Replicate-11		
F	Replicate-6		Replicate-12		
G					
H					

5. Seal with Microseal 'B' and pulse spin plate (3 seconds at 280 x g).

6. Shake plate at 1600 rpm for 1 minute. Ensure that all beads are re-suspended and that no sample has splashed into either the sides of the wells or the seal.
 - If there are beads that have not re-suspended, unseal plate and pipette to mix. Re-seal plate.
 - After shake, if liquid is on the sides of the well, pulse spin plate again and repeat shake at 1800 rpm for 1 minute.

7. Ensure that **TAG55** program is correct on thermocycler:

Heated Lid: 100 °C

Volume: 50 µL

Step	Temperature	Time
1	55 °C	5 minutes
Hold	12°C	Hold

8. Place sealed sample plate onto thermocycler and start program **TAG55**. Program is approximately 6-8 minutes.
 9. During incubation, pipette out ST2 buffer into a strip tube. For 12 samples, add at least 25 µL of ST2 buffer to 6 wells of a strip tube.
 10. Remove samples immediately after sample temperature has reached 12°C. Leave samples at room temperature for 2 minutes.
 11. Remove Microseal 'B' and add 10 µL of ST2 to each sample, pipette mix 3-5x.
 12. Seal sample plate with Microseal 'B'. Shake plate at 1600 rpm for 1 minute and then incubate at room temperature for an additional 4 minutes.
 - If splashes occur, pulse spin plate (3 seconds at 280 x g).
- Note:** Steps 13 - 18 are sensitive to drying out.
13. Place sample plate on magnetic (strip magnet for PCR plates) stand for 1 minute.
 14. Remove Microseal 'B'.
 15. Remove supernatant and discard. Do not allow bead pellet to dry out.
 16. Remove plate from magnet. Using a multi-channel pipette, add 100 µl of TWB to each sample. Seal sample plate with Microseal 'B'.
 17. Shake plate at 1600 rpm for 1 minute.
 - If splashes occur, pulse spin plate (3 seconds at 280 x g).
 - If pellet is still present after 1600 rpm shake or spin, then shake plate at 1800 rpm for 1 minute.



18. Repeat steps **13 - 17** two times, for a total of 3 washes. On final wash, leave plate on magnet with wash buffer still in sample plate.

Safe stopping point: Samples are stable at room temperature with buffer for up to 80 minutes.

2 Library PCR

- Hands on time: approximately 10 minutes
- Total time: approximately 45 minutes

2.1 Reagent and Preparation

Reagent	Preparation
Molecular Grade Water	<ul style="list-style-type: none">• Make an aliquot for use (at least 300 µL) or continue use of previous aliquot• May be kept at room temperature
EPM	<ul style="list-style-type: none">• Thaw on ice• Invert to mix and spin down
UDI PCR Index Set A	<ul style="list-style-type: none">• Thaw at room temperature• Spin down• Puncture wells to be used (Recommend columns 1 and 2)

2.2 Procedure Steps

1. Prepare PCR Master Mix for 12 samples in a 1.5 mL Eppendorf tube in the order listed:

Reagent	Volume per sample (µL)	Volume (12 Samples)* (µL)
Molecular Grade Water	20	264
EPM	20	264
Total	40	528

*Includes 10% overage

2. Briefly vortex and spin down PCR Master Mix.
3. Pipette master mix into a strip tube (approximately 82 µL per tube in 6 tubes). Samples are still sensitive to drying, so perform this step prior to removing supernatant for samples. Do not allow sample pellet to dry out.
4. Ensure UDI PCR Index Set A plate is ready for use.
5. Remove and discard supernatant from samples.
6. Using a 20 µL pipette, remove and discard any residual supernatant.
7. Remove plate from magnet.

8. Add 40 µL PCR Master Mix to each sample.
9. Add 10 µL of Indices to each sample. Each sample must have a different Index pair from a different well in the UDI PCR Index Set plate. Indices used will be recorded at Step **16**.

Note: Each well of the Index plate has a different pair of indices, so each sample must have indices from a different well. Although it is recommended to use the indices in the first two columns (see **Figure 2**), any indices on the plate may be used. Indices used will be recorded in the *SampleSheet, SMB NFE, 1000000095063*.

For more information see **Appendix A and B**.

Figure 2: Recommended Index Usage

	1	2	3	...	12
A	A1		A2		
B	B1		B2		
C	C1		C2		
D	D1		D2		
E	E1		E2		
F	F1		F2		
G					
H					

Note: Sample labels (A1-F2) indicate location from Index plate for use in the above sample plate.

10. Seal plate with Microseal 'B'
11. Shake plate at 1800 rpm for 30 seconds. After shake, check plate for any bead clumping, if any clumps exist pipette up and down to re-suspend.
12. Pulse spin plate (3 seconds at 280 x g).
13. Shake plate at 1600 rpm for 30 seconds.

14. Ensure that **EPMAMP9** program is correct on thermocycler:

Heated Lid: 100 °C

Volume: 50 µL

Step	Temperature	Time
1	72°C	3 minutes
2	98°C	3 minutes
3	98°C	20 seconds
4	60°C	30 seconds
5	72°C	1 minute
6	Repeat steps 3-5 8 times, 9 cycles total	
7	72°C	3 minutes
Hold	10°C	

15. Place sealed sample plate onto thermocycler and start program **EPMAMP9**. Program is approximately 35 minutes.

16. Record indices used in *SampleSheet*, *SMB NFE*, 1000000095063, if wells A1-F1 and A2-F2 were used, these indices are already present in the SampleSheet. If other indices were used or if they were used in a different order, see **Appendix A and B**.

Safe stopping point: Reactions are stable at 10°C on the thermocycler or stored at 2°C-8°C for 72 hours.

3 PCR Clean Up and Pooling

- Hands on time: approximately 35 minutes
- Total time: approximately 35 minutes

3.1 Reagent Preparation

Reagent	Preparation
SPB	Bring to room temperature for at least 30 minutes prior to use
EtOH	Make fresh 80% by adding 8 mL of 100 % EtOH to 2 mL of Molecular Grade Water
Molecular Grade Water	<ul style="list-style-type: none">• Make an aliquot for use (at least 1 mL)• May be kept at room temperature

Reagent	Preparation
RSB	N/A, may be kept at room temperature
dsDNA Broad Range Qubit kit	<ul style="list-style-type: none"> Remove items from storage and bring to room temperature for at least 30 minutes

3.2 Procedure Steps

1. Label a new MIDI plate with **TiCU-1**, initials, and date.

2. Remove sample plate from thermal cycler.

Note: Beads may have settled during PCR, this is to be expected and will not impact results.

3. Spin down for 1 minute at 280 x g, ensure that all liquid is at the bottom of the well.

4. Shake sample plate at 1800 rpm for 1 minute.

5. Place sample plate on magnetic (strip magnet for PCR plates) stand for 1 minute.

6. Add the following reagents in the order listed to **TiCU-1** plate. Molecular Grade Water and SPB may be added to troughs. Use a multi-channel pipette and use **Figure 3** for reference.

Reagent	Volume per sample (μl)
The following will go into columns 1 and 3:	
Molecular Grade Water	77
SPB	88*

*When pipetting SPB, dispense slowly and wait 5 seconds for residual SPB to reach the bottom of the tip. Wash tips in mixture already present in wells by pipette mixing briefly.

Reaction from TiLP-1	45
Total	210

The following will go into columns 5 and 7:

SPB	20
-----	----

Figure 3: TiCU-1 Plate Layout Part 1

	1	2	3	4	5	6	7	...	12
A	Replicate-1 SPB+H2O		Replicate-7 SPB+H2O		SPB		SPB		
B	Replicate-2 SPB+H2O		Replicate-8 SPB+H2O		SPB		SPB		

	1	2	3	4	5	6	7	...	12
C	Replicate-3 SPB+H2O		Replicate-9 SPB+H2O		SPB		SPB		
D	Replicate-4 SPB+H2O		Replicate-10 SPB+H2O		SPB		SPB		
E	Replicate-5 SPB+H2O		Replicate-11 SPB+H2O		SPB		SPB		
F	Replicate-6 SPB+H2O		Replicate-12 SPB+H2O		SPB		SPB		
G									
H									

7. Seal **TiCU-1** plate with Microseal 'B'. Shake plate at 1800 rpm for 1 minute and then incubate at room temperature for an additional 4 minutes.
8. Place sample plate on magnetic (peg magnet for MIDI plates) stand for 5 minutes.
9. Transfer 200 µL supernatant of each sample to SPB containing columns (5 and 7) of **TiCU-1** plate. See **Figure 4**.

Figure 4: TiCU-1 Plate Layout Part 2

	1	2	3	4	5	6	7	...	12
A	Used SPB		Used SPB		Replicate-1		Replicate-7		
B	Used SPB		Used SPB		Replicate-2		Replicate-8		
C	Used SPB		Used SPB		Replicate-3		Replicate-9		
D	Used SPB		Used SPB		Replicate-4		Replicate-10		
E	Used SPB		Used SPB		Replicate-5		Replicate-11		
F	Used SPB		Used SPB		Replicate-6		Replicate-12		
G									
H									

10. Seal sample plate with Microseal 'B'. Shake plate at 1800 rpm for 1 minute and then incubate at room temperature for an additional 4 minutes.
11. Place **TiCU-1** plate on magnetic (peg magnet for MIDI plates) stand for 5 minutes.
12. Leave plate on magnet. Remove and discard supernatant using multi-channel 200 µL pipette.



13. Leaving plate on magnet, add 200 µL 80% EtOH to each sample.
14. Incubate at room temperature for 30 seconds.
15. Repeat steps **12 - 14** one more time, for a total of two washes.
16. Remove and discard supernatant, incubate plate on magnet at room temperature for 5 minutes.
17. During 5-minute incubation, using a 20 µL multi-channel pipette, remove any residual EtOH solution from sample wells.
18. Remove from magnet.
19. Add 17 µL of RSB to sample wells.
20. Seal plate with Microseal 'B'.
21. Pulse spin down plate, 280 x g for 3 seconds.
22. Shake plate at 1800 rpm for 1 minute.
23. Incubate at room temperature 2 additional minutes.
24. Place plate on magnetic (peg magnet for MIDI plates) stand for 2 minutes.
25. Label a new PCR plate with **TiLP-2**, initials, date, and plate ID.
26. Leave **TiCU-1** plate on magnet, remove 15 µL eluent from each sample and add to empty wells of **TiLP-2** plate.

Note: Safe stopping point indicated after step **33** may be used before or after quantification

27. Quantify library prep samples by using a dsDNA Broad Range Qubit kit. Reference WI, Quantification for Sample Prep, 15024021 for instructions on how to use the Qubit kit. One reading per sample is sufficient.

Example Qubit Working solution: 12 DNA samples and 2 standards:

15 µL of BR Reagent + 2985 µL of BR Buffer = 3000 µL of Working Solution

28. Record Library Preparation Yield values in *SampleSheet*, SMB NFE, 1000000095063. For more information, see **Appendix A**.
29. Samples must have a concentration of at least 33.3 ng/µl (500 ng total sample) to be considered passing. A certain number of samples must be passing to proceed. See **Table 1**.

Table 1: Library Prep Yield Requirements

Reagent Being Tested	Number of Samples below 33.3 ng/µl	
SMB (15039631, 20033748, or 20029947)	≤ 2	Proceed, go to step 31



30. If insufficient number of samples pass library preparation yield, create a folder in \\ussd-prd-isi6\fare\Runs\Production\NextSeq labeled with date, initials and plate ID. Save SampleSheet to newly created folder, as a .csv file. A report will be generated, go to **Data Analysis and Reporting**.
31. Pool 10 samples only, 2 samples will not be used for enrichment, but library preparation yields will still be recorded. If any samples are below 33.3 ng/µl, do not pool those samples. Even if all samples are above 33.3 ng/µl, only 10 samples will be pooled.
32. Label a new PCR plate with **TiHYB**, initials, date, and plate ID.
33. Pool samples by volume, add 3 µl of each chosen sample to one well of **TiHYB** plate, for a total of 30 µL. This plate will be used in the hybridization.

Safe stopping point: **TiLP-2** and **TiHYB** are both stable at -25°C to -15°C for 30 days. **TiHYB** is stable for 30 days, minus time of storage for **TiLP-2**.

4 Hybridization

- Hands on time: approximately 20 minutes
- Total time: approximately 145 minutes

4.1 Reagent Preparation

Reagent/Consumable	Preparation
TruSight One oligo panel	Thaw, vortex, quick spin to ensure no reagent is present on lid
NHB1 or NHB2	<ul style="list-style-type: none">• Only one hybridization buffer may be used, NHB1 and NHB2 may not be used in the same run• Thaw, vortex, quick spin to ensure that no reagent is present on lid• Warm up by placing on Hybex block heated to 50°C for approximately 10-15 minutes• Pipette up and down to ensure no precipitates are present.• Check for precipitates, if precipitates are present vortex and continue warming until dissolved.
EHB2	Vortex, quick spin (just enough to remove reagent from lid), check for precipitates, if precipitates are present vortex and continue warming at room temperature until dissolved.

4.2 Procedure Steps

1. Bring **TiHYB** plate to room temperature.

2. Spin down for 1 minute at 280 x g.
3. Add the following reagents, in the order listed, to the sample in the **TiHYB** plate. Mix by pipetting 10X, slowly to avoid bubbles.

Reagent	Volume (1 Sample) (µL)
TruSight One oligo panel	10
NHB1 or NHB2	50
EHB2	10
Total	100

Note: Do not make master mix containing either NHB1 or NHB2 and EHB2

4. Set pipette to 80 µL and pipette 10x to mix.
5. Seal with Microseal 'B', spin down at 280 xg for 1 minute.
6. Ensure that **TISMBHYB** program is correct on thermocycler:

Heated Lid: 100 °C

Volume: 100 µL

Step	Temperature	Time
1	95°C	5 minutes
2	94°C	1 minute
3	Increment: Decrease 2°C per cycle 15 times (for a total of 16 cycles) to reach 62°C	
4	62°C	90 minutes
Hold	25°C	

7. Put **TiHYB** on thermocycler, start program **TISMBHYB**.

Note: If using a thermocycler with a maximum volume of 50 µL, input volume as 50 µL otherwise input 100 µL, actual reaction volume is 100 µL, entire reaction must be used.

8. Set Hybex incubator to 62°C prior to **Capture** step.

Safe stopping point: Hybridization reaction is stable at room temperature up to 16 hours.

Note: Do not keep hybridization reaction at temperatures below room temperature.

5 Capture

- Hands on time: approximately 40 minutes

- Total time: approximately 85 minutes

5.1 Reagent Preparation

Reagent/Consumable Preparation

SMB	<ul style="list-style-type: none">• Remove from storage at least 30 minutes before use.• Vortex thoroughly directly before use and ensure that beads are re-suspended evenly
EEW	<ul style="list-style-type: none">• Remove from storage at least 30 minutes before us.• Warm to 62°C by placing tube on top of Hybex block for about 30 minutes.• Keep warm throughout procedure by leaving on 62°C Hybex block in between washes.
ET2	Bring to room temperature, vortex, spin down.
EE1	Thaw at room temperature, vortex, spin down.
HP3	Thaw at room temperature, vortex, spin down.

5.2 Procedure Steps

1. Label a new MIDI plate with **TiCAP**, initials, and date.
2. Remove **TiHYB** plate from thermocycler and spin down (280 x g for 1 minute).
3. Transfer 250 µL of SMB to well A1 of **TiCAP** plate.
4. Transfer entire hybridization reaction (100 µL, some evaporation may have occurred) from **TiHYB** to SMB in **TiCAP**.
5. Seal plate with Microseal 'B', shake at 1200 rpm for 4 minutes.
6. Place **TiCAP** on lab bench at room temperature for 5 minutes.
7. Place **TiCAP** on Hybex set to 62°C for 15 minutes.
8. Immediately quick spin plate (280 xg for 3 seconds).
9. Immediately place plate on magnet for 2 minutes, or until clear.

Note: To keep track of washes, it is recommended to save supernatant in plate in separate wells, see **Figure 5** below:

10. Remove supernatant, 200 µL at a time, using 200 µL pipette.
11. Remove from magnet, add 200 µL of warmed EEW to sample.
12. Seal plate with Microseal 'B', shake at 1800 rpm for 4 minutes.
13. Incubate on Hybex for 5 minutes at 62°C.

14. Repeat steps 9 - 13 two times for a total of 3 washes, one additional wash will follow in steps 15 - 20.

Figure 5: Hybridization Wash TiCAP Plate Layout

	1	2	3	...	9	10	11	12
A	Reaction				Wash Supernatant 3	Wash Supernatant 2	Wash Supernatant 1	Reaction Supernatant
B								
...								
H								

15. Immediately place plate on magnet for 2 minutes, or until clear.
16. Remove supernatant, 200 µL at a time, using 200 µL pipette.
17. Remove from magnet, add 200 µL of warmed EEW to sample.
18. Seal plate with Microseal 'B', shake at 1800 rpm for 4 minutes.
19. Transfer entire sample (EEW and beads) to new well. See Figure 6: Fourth EEW Wash.
20. Seal plate with Microseal 'B'.
21. Incubate on Hybex for 5 minutes at 62°C.

Figure 6: Fourth EEW Wash Wash TiCAP Plate Layout

	1	2	...	7	8	9	10	11	12
A	Reaction			Reaction and Beads		Wash Supernatant 3	Wash Supernatant 2	Wash Supernatant 1	Reaction Supernatant
B									

22. During 5-minute incubation, prepare the following elution mixture:

Reagent	Volume (1 Sample) (µL)
EE1	28.5
HP3	1.5
Total	30



Note: Mixture may be left at room temperature

23. Immediately place plate on magnet for 2 minutes, or until clear.
24. Remove supernatant, 200 µL at a time, using 200 µL pipette.
25. Seal plate with Microseal 'B'.
26. Quick spin plate, 280 xg for 3 seconds.
27. Place on magnet and use 20 µL pipette to remove any residual EEW.
28. Remove from magnet.
29. Add 23 µL of EE1/HP3 to sample beads.
30. Seal **TiCAP** with Microseal 'B', shake at 1800 rpm for 2 minutes.
31. Incubate at room temperature for an additional 2 minutes.
32. Spin down plate at 280 xg for 1 minute.
33. Place on magnet for 2 minutes, or until clear.
34. Label a new PCR plate with **TiPCR-2**, initials, date, and plate ID.
35. Remove 21 µL of eluent and add to **TiPCR-2** plate.
36. Add 4 µL of ET2 to sample in **TiPCR-2** plate. Pipette to mix.
37. Immediately proceed to **Second PCR and Clean Up**.

6 Second PCR and Clean Up

- Hands on time: approximately 40 minutes
- Total time: approximately 75 minutes

6.1 Reagent Preparation

Reagent/Consumable Preparation

PPC	Thaw on ice
EPM	Thaw on ice
SPB	<ul style="list-style-type: none">• Bring to room temperature• Vortex thoroughly before use
Ethanol	Make fresh 80% by adding 800 µL of 100 % EtOH to 200 µL of Molecular Grade Water

6.2 Procedure Steps

1. Add 5 µL of PPC to sample in **TiPCR-2** plate.
2. Add 20 µL EPM to sample in **TiPCR-2** plate. Pipette well to mix.
3. Seal with Microseal 'A', spin at 280 xg for 1 minute.
4. Ensure that **AMP12** program is correct on thermocycler:

Heated Lid: 100 °C

Volume: 50 µL

Step	Temperature	Time
1	98°C	30 seconds
2	98°C	10 seconds
3	60°C	30 seconds
4	72°C	30 seconds
5	Repeat steps 2-4 11 times, 12 cycles total	
6	72°C	5 minutes
Hold	4°C	

5. Place sealed sample plate onto thermocycler and start program **AMP12**. Program is approximately 30 minutes.

Safe stopping point: Seal plate with Microseal 'B' and store at 2°C to 8°C for up to 2 days, alternatively leave on thermocycler at 4°C hold overnight.

6. Label a new MIDI plate with **TiCU-2**, initials, and date.
7. Spin down **TiPCR-2** at 280 xg for 1 minute.
8. Add 40.5 µL of SPB to sample well of **TiCU-2** plate.
9. Remove seal of **TiPCR-2** plate and transfer 45 µL to well of **TiCU-2** containing SPB.
10. Seal with Microseal 'B', shake at 1800 rpm for 1 minute, and incubate at room temperature for an additional 5 minutes.
11. Spin plate at 280 x g for 1 minute.
12. Place plate on magnet for 2 minutes or until clear. Remove and discard supernatant.
13. Leave on magnet, add 200 µL of 80% EtOH without disturbing pellet.

14. Incubate at room temperature, on magnet for 30 seconds.
15. Remove and discard supernatant.
16. Repeat steps **13-15** one time, for a total of 2 washes.
17. Remove residual ethanol using a 20 µL pipette.
18. Air dry sample for 5 minutes.
19. Remove plate from magnet, add 32 µL of RSB.
20. Seal with Microseal 'B', shake at 1800 rpm for 1 minute.
21. Spin plate at 280 xg for 1 minute.
22. Place on magnet for 2 minutes or until clear.
23. Label a new PCR plate with **TiFinal**, initials, date, and plate ID.
24. Remove seal, transfer 30 µL of supernatant to **TiFinal** plate.

Note: Safe stopping point indicated after step **27** may be used before or after quantification

25. Quantify enrichment sample in triplicate by using ~~a~~/dsDNA Broad Range Qubit kit. Reference WI, Quantification for Sample Prep, 15024021 for instructions on how to use the Qubit kit.

Example Qubit Working solution: 1 DNA sample, in triplicate, and 2 standards:

6 µL of BR Reagent + 1194 µL of BR Buffer = 1200 µL of Working Solution

26. Sample must be above 0.5 ng/µL to move on to enrichment. Record results in SampleSheet.
27. If yield is below 0.5 ng/µL, create a folder in \\ussd-prd-isi06\fare\Runs\Production\NextSeq labeled with date, initials and plate ID. Save SampleSheet to newly created folder. A report will be generated go to **Data Analysis and Reporting**.

Safe stopping point: If stopping, seal plate with Microseal 'B' and store at -25°C to -15°C for up to seven days.

7 Sequencing Preparation

- Hands on time: approximately 30 minutes
- Total time: approximately 30 hours



7.1 Reagent Preparation

Reagent	Preparation
Molecular Grade Water	<ul style="list-style-type: none">• Make an aliquot for use (at least 50 µL) or continue use of previous aliquot• May be kept at room temperature
HT1	Thaw, keep chilled.
NextSeq 500/550 v2 Cartridge (300 cycle)	Thaw in water bath (approximately 90 minutes) or overnight at 4°C
NextSeq 500/550 v2 Buffer Cartridge	N/A
NextSeq 500/550 v2/2.5 Flow Cell	Bring to room temperature
HP3	Create 0.2 NaOH from 2.0 N stock by adding 2 µL of HP3 to 18 µL of Molecular Grade Water
1 M Tris-HCl pH 8	Create 200 mM Tris-HCl from 1 M stock by adding 4 µL 1 M Tris-HCl to 16 µL Molecular Grade Water
RSB	N/A, may be kept at room temperature

7.2 Procedure Steps

1. Prepare equipment and reagents specific to the NextSeq 500/550 as indicated in WI, NextSeq Reagent Sequencing QC, 15049213.
2. Dilute enrichment reaction (**TiFinal**) to 4 nM with final volume of at least 50 µL, using RSB.

A. Concentration in nM:

$$\frac{\text{Concentration in } \frac{\text{ng}}{\mu\text{L}} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times 350 \text{ bp}} = \text{Molarity in nM}$$

B. DNA volume needed:

$$\frac{\text{Final volume in } \mu\text{L} \times 4 \text{ nM}}{\text{Initial Molarity in nM}} = \text{DNA Volume needed in } \mu\text{L}$$

C. RSB volume:

$$\text{Final Volume in } \mu\text{L} - \text{DNA Volume needed in } \mu\text{L} = \text{RSB Volume needed in } \mu\text{L}$$

Example:

A. Concentration in nM = $((20) * (10^6)) / (660 * 350)$

Concentration in nM = $20000000 / 231000$

Concentration in nM = 86.6 nM

B. DNA volume needed = $(50 \mu\text{L} \times 4 \text{ nM}) / 86.6 \text{ nM}$

DNA volume needed = 2.3 μL

C. RSB volume = $50 \mu\text{L} - 2.3 \mu\text{L}$

RSB volume = 47.7 μL

Note: Do not pipette less than 2 μL , if required volume of DNA is less than 2 μL , scale up final volume. Volumes will be different based off of initial concentration.

3. Denature 4 nM Library by adding 5 μL of Library from Step 2 and 5 μL of 0.2 N NaOH.
4. Vortex and quick spin (just enough to remove reagent from lid).
5. Incubate at room temperature for 5 minutes, then immediately add 5 μL of 200 mM Tris-HCl.
6. Vortex and quick spin (just enough to remove reagent from lid).
7. Dilute Library to 20 pM by adding 985 μL of chilled HT1, vortex and quick spin (just enough to remove sample from lid).
8. Dilute Library to loading concentration. A loading concentration of 1.0 pM is recommended but may be adjusted to achieve recommended cluster density, make at least 1.3 mL:

A. DNA volume needed:

$$\frac{\text{Final Volume in } \mu\text{L} \times \text{Final Concentration in pM}}{20 \text{ pM}} = \text{Volume needed of 20 pM DNA}$$

B. HT1 volume needed:

$$\text{Final Volume in } \mu\text{L} - \text{DNA Volume} = \text{HT1 Volume}$$

Example:

A. DNA volume needed = $(1300 \mu\text{L} \times 1.0 \text{ pM}) / 20 \text{ pM}$

DNA volume needed = 65 μL

B. HT1 volume = $1300 \mu\text{L} - 65 \mu\text{L}$

HT1 volume = 1235 μL

Note: Recommended raw cluster density range is 160-220 k/mm², adjust loading concentration as necessary.

9. Load and run NextSeq 500/550 as indicated in NextSeq 500/550 as indicated in WI, NextSeq Reagent Sequencing QC, 15049213.
10. Save to appropriate folder.
11. Recipe: Paired End: 125+125+10+10



12. Run will take approximately 30 hours.
13. Go to **Data Analysis and Reporting** before run completes to allow automated analysis to begin.

8 Data Analysis and Reporting

- Hands on time: approximately 10 minutes
- Total time: approximately 6 hours

8.1 Data Analysis

1. Ensure that the following information exists in the SampleSheet:
 - A. Experiment Name: to be filled in by the analyst
 - B. Date: to be filled in by the analyst
 - C. Investigator: to be filled in by the analyst, use accessible email address to receive notifications and data
 - D. Project: TiSMBanalysis, exists within released document, do not edit.
 - E. Library Prep Material: leave blank.
 - F. Enrichment Material: to be filled in by the analyst, include both Material number and Batch Number with no spaces, use this format: MNxxxxxx_BNxxxxxx.
 - 1) TC#-SMB,STRPTVDN MAGBDS,FIN RGT 1.2 mL, MN15039631
 - 2) SO#-SMB, Streptavidin Magnetic Beads MN20029947
 - 3) SO#-SMB, STRPTVDN MAGBDS, uHT, MN20033748

Note: See **Appendix A** for more information on SampleSheet.

2. Save SampleSheet in appropriate run folder as a .csv file.

Note: SampleSheet must be saved as "SampleSheet.csv"

- A. If run failed at either yield step, create a folder in the same location as if the run was being sequenced. Label the folder with the date, initials, and plate id ensuring there are no spaces in the folder name.

Example: \\ussd-prd-isi06\fare\Runs\Production\NextSeq\20181030_AS_plateID

If disposition is Invalid or Fail, follow *Procedure, Out of Specification Procedure, 15026737*.



- B. If run is being sequenced, use run folder.
- 3. When Analysis starts, an email will be sent to the address in the SampleSheet.
- 4. When analysis completes, an email that includes the PDF report of results will be sent to the address in the SampleSheet.
 - A. If run failed a Library Prep or Enrichment Yield, the analysis will still provide a report with either Invalid or Failed results.
 - B. If run was sequenced, analysis will take approximately 6 hours after sequencing run is complete.

Note: If SampleSheet is not saved in the appropriate folder, analysis will not start. SampleSheet may be saved in appropriate folder as soon as the folder is created, either by the operator (failed yield values, no sequencing) or by the NextSeq.

8.2 Data Reporting

- 1. Once analysis is complete, a PDF report will be generated. It can be found in the analysis folder in the run folder, or created folder.
- 2. PDF will report individual sample results, disposition calls for each metric, and a final disposition.

Note: This assay allows for a reaction pass rate of $\geq 90\%$. This means that 1 sample may fail for final enrichment results. Print, sign and date form in provided spaces.

- 3. If disposition is Invalid or Fail, follow *Procedure, Out of Specification Procedure, 15026737*.
- 4. For additional information on specification see *Engineering Report, Titanium QC Full Functional Assay, 1000000069709* and *SMB NFE spec setting engineering report 1000000103688*.

8.3 Non-NFE Testing – For MN 15039631 Only

If MN 15039631 fails the NFE assay OOS with no assignable cause, do not initiate an NCR. The material is not NFE Qualified and must be tested using WI 15041444, per MCS DIR 1503961. Only MN 15039631 can be tested on an alternate workflow. All other SMB MNs tested on this WI must pass NFE testing to be released.

Appendix A SampleSheet Instructions

A.1 The SampleSheet

- 1. SampleSheet, SMB NFE, 1000000095063 should be used for this WI.
- 2. The Samplesheet must be saved as a .csv file.
- 3. Only the following cells may be edited: B4-6, B10-B22, A49-H60, Row 8 and 9 after column B.



4. The Project must be "TiSMBanalysis" in order for the automated analysis to run.
5. Manifest information in cells B35 and B36 are specific to the TruSight One probe pool.

A.2 Input analyst email and tested materials

1. In order for the analyst to receive emails about; analysis start, analysis complete, analysis error, and to received PDF report, the analyst must enter their email in cell B6.
2. PDF report will be saved in the analysis folder found inside of the run folder whether or not the PDF is emailed.
3. Tested reagents must be recorded in the SampleSheet.
 - A. Do not leave spaces or use special characters, format should be: MNxxxxxx_BNxxxxxx.
 - B. If multiple reagents were tested, fill out information for all reagents use a different cell for each reagent.
 - C. Only list materials being tested.
 - 1) Correct

	A	B	C	D
8	Library Prep Material	MN20024592	_BN20302308	
9	Enrichment Material	MN20024596	_BN20302310	

2) Incorrect (spaces)

	A	B	C	D
8	Library Prep Material	MN20024592	BN20302308	
9	Enrichment Material	MN20024596	BN20302310	

3) Incorrect (commas, multiple reagents in one cell)

	A	B	C	D	E	F
8	Library Prep Material	MN20024592	_BN20302308, MN12345	_BN12345		
9	Enrichment Material					

A.3 Indices

1. Indices listed in SampleSheet must reflect actual indices used and must correlate to correct sample number.
2. SampleSheet, SMB NFE 1000000095063 contains index information for wells A1-F1 and A2-F2.



- A. If different wells of the index plate were used, use Appendix B to fill out the correct information.
- B. Once a determination of samples to be dropped before enrichment is made, delete the corresponding rows of these samples.
- C. Sample_Plate, Sample_Well, Sample_Project, and Description do not need to be filled out.

Appendix B : Index Sequences Set A

Index Plate Well	I7 Index ID	I7 Index Seq	I5 Index ID	I5 Index Seq
A01	UDP0001	GAAC TGAG CG	UDP0001	CGCT CCAC GA
B01	UDP0002	AGGT CAGA TA	UDP0002	TATC TTGT AG
C01	UDP0003	CGT CTC ATAT	UDP0003	AGCT ACTA TA
D01	UDP0004	ATT CCATA AG	UDP0004	CCAC CAGG CA
E01	UDP0005	GAC GAGA TTA	UDP0005	AGG ATAAT GT
F01	UDP0006	AA CA TGCG GC	UDP0006	ACA AGTGG AC
G01	UDP0007	CT AGTG CTCT	UDP0007	TACT GTTCCA
H01	UDP0008	GAT CAAGG CA	UDP0008	ATTA ACAAGG
A02	UDP0009	GA CTGAG TAG	UDP0009	CA CTAT CAAC
B02	UDP0010	AG TCAG ACGA	UDP0010	TG TC GCT GGT
C02	UDP0011	CC GTAT GTTC	UDP0011	AC AGTGTAT G
D02	UDP0012	GAG TCATA GG	UDP0012	AG CGCC ACAC
E02	UDP0013	CT TGCC ATT A	UDP0013	CCTT CGTG AT
F02	UDP0014	GA AGCG GCAC	UDP0014	AG TAGAG CCG
G02	UDP0015	TCC ATTG CCG	UDP0015	TC GTGC ATT C
H02	UDP0016	CG GTTAC CGC	UDP0016	CT ATAG TCTT
A03	UDP0017	GAGA ATGG TT	UDP0017	TT GCTGCC GA
B03	UDP0018	AG AGGCA ACC	UDP0018	CC ATCATT AG
C03	UDP0019	CC ATCATT AG	UDP0019	AG AGGCA ACC
D03	UDP0020	GAT AGGCC GA	UDP0020	GCC ATGT GCG
E03	UDP0021	AT GGTT GACT	UDP0021	AGG ACAGG CC
F03	UDP0022	TAT TGCG CTC	UDP0022	CCT AACAC AG
G03	UDP0023	AC GCTT GTTT	UDP0023	AC GTT CCTT A
H03	UDP0024	TT CTAC ATAC	UDP0024	TT ACAG TTAG
A04	UDP0025	AAC CATAG AA	UDP0025	CC ATCT CGCC
B04	UDP0026	GG TTGCG AGG	UDP0026	TT GCTCT ATT
C04	UDP0027	TA AGC ATCCA	UDP0027	AAT GGATT GA
D04	UDP0028	ACC AC GACAT	UDP0028	CC GCATAC GA
E04	UDP0029	GCC GCACT CT	UDP0029	CG AGGT CGGA
F04	UDP0030	CC ACCA GAGG CA	UDP0030	AT TC CATA AG
G04	UDP0031	GT GACAC GCA	UDP0031	GT CC GTAA GC
H04	UDP0032	AC AGTGTAT G	UDP0032	CC GTAT GTTC



A05	UDP0033	TGATTATACG	UDP0033	TGTAATCGAC
B05	UDP0034	CAGCCGCGTA	UDP0034	CACGGCTAGT
C05	UDP0035	GGTAACTCGC	UDP0035	TCACCAACTT
D05	UDP0036	ACCGGCCGTA	UDP0036	AATATTGCCA
E05	UDP0037	TGTAATCGAC	UDP0037	CGCGGTGATC
F05	UDP0038	GTGCAGACAG	UDP0038	ACGGATGGTA
G05	UDP0039	CAATCGGCTG	UDP0039	TTCCTACAGC
H05	UDP0040	TATGTAGTC	UDP0040	CATTAGTGCG
A06	UDP0041	ACTCGGCAAT	UDP0041	TTCAGTTGTC
B06	UDP0042	GTCTAATGGC	UDP0042	CCTGACCAC
C06	UDP0043	CCATCTGCC	UDP0043	AACCATAAGAA
D06	UDP0044	CTGCGAGCCA	UDP0044	TGGCCGGATT
E06	UDP0045	CGTTATTCTA	UDP0045	AACCTTATGG
F06	UDP0046	AGATCCATTA	UDP0046	TGGTAGAGAT
G06	UDP0047	GTCCTGGATA	UDP0047	TTCGCCACCG
H06	UDP0048	CAGTGGCACT	UDP0048	CCTATTGTTA
A07	UDP0049	AGTGTTCAC	UDP0049	CGTGTACCAAG
B07	UDP0050	GACACCATGT	UDP0050	TACACGTTGA
C07	UDP0051	CCTGTCTGTC	UDP0051	TCACAACAGT
D07	UDP0052	TGATGTAAGA	UDP0052	AAGGACGAC
E07	UDP0053	GGAATTGTAA	UDP0053	AGGATGTGCT
F07	UDP0054	GCATAAGCTT	UDP0054	TGCGACGGAA
G07	UDP0055	CTGAGGAATA	UDP0055	AGTGGTTAAG
H07	UDP0056	AACGCACGAG	UDP0056	TATCCGAGGC
A08	UDP0057	TCTATCCTAA	UDP0057	CCAGTCGACG
B08	UDP0058	CTCGCTTCGG	UDP0058	TTGACTAGTA
C08	UDP0059	CTGTTGGTCC	UDP0059	AACGGTCTAT
D08	UDP0060	TTACCTGGAA	UDP0060	CTGGAACGT
E08	UDP0061	TGGCTAATCA	UDP0061	CTACATGCCT
F08	UDP0062	AACACTGTTA	UDP0062	TGAGACTTGC
G08	UDP0063	ATTGCGCGGT	UDP0063	GCGGAGCCAA
H08	UDP0064	TGGCGCGAAC	UDP0064	AGTATCAGTT
A09	UDP0065	TAATGTGTCT	UDP0065	TATGCCCTAC
B09	UDP0066	ATACCAACGC	UDP0066	CGCAGCAATT
C09	UDP0067	AGGATGTGCT	UDP0067	GGAATTGTAA
D09	UDP0068	CACGGAACAA	UDP0068	GTGCTAGGTT
E09	UDP0069	TGGAGTACTT	UDP0069	TCCACACAGA
F09	UDP0070	GTATTGACGT	UDP0070	TTGGAATTCC
G09	UDP0071	CTTGTACACC	UDP0071	AAGCGCGCTT
H09	UDP0072	ACACAGGTGG	UDP0072	ACAACGCTCA
A10	UDP0073	CCTGCGGAAC	UDP0073	AGCCTATGAT

B10	UDP0074	TTCATAAGGT	UDP0074	CCTTCTAAC
C10	UDP0075	CTCTGCAGCG	UDP0075	TACATCCATC
D10	UDP0076	CTGACTCTAC	UDP0076	TGACGGCCGT
E10	UDP0077	TCTGGTATCC	UDP0077	GTAAGCAACG
F10	UDP0078	CATTAGTGCG	UDP0078	TATGTAGTCA
G10	UDP0079	ACGGTCAGGA	UDP0079	AACGAGGCCG
H10	UDP0080	GGCAAGCCAG	UDP0080	CGGATGCTT
A11	UDP0081	TGTCGCTGGT	UDP0081	AGTCAGACGA
B11	UDP0082	ACCGTTACAA	UDP0082	TCGCTATGAG
C11	UDP0083	TATGCCCTAC	UDP0083	TAATGTGTCT
D11	UDP0084	ACAAGTGGAC	UDP0084	AACATCGCGC
E11	UDP0085	TGGTACCTAA	UDP0085	AGTACTCATG
F11	UDP0086	TTGGAATTCC	UDP0086	GTATTGACGT
G11	UDP0087	CCTCTACATG	UDP0087	AGGAGGTATC
H11	UDP0088	GGAGCGTGTA	UDP0088	ACTTACGGAT
A12	UDP0089	GTCCGTAAGC	UDP0089	AAGATACACG
B12	UDP0090	ACTTCAAGCG	UDP0090	TTCATGGTTC
C12	UDP0091	TCAGAAGGCG	UDP0091	TATGATGGCC
D12	UDP0092	GCGTTGGTAT	UDP0092	GGAAGTATGT
E12	UDP0093	ACATATCCAG	UDP0093	ATTGCACATA
F12	UDP0094	TCATAGATTG	UDP0094	CACCTTAATC
G12	UDP0095	GTATTCCACC	UDP0095	TTGTCTACAT
H12	UDP0096	CCTCCGTCCA	UDP0096	CACCGATGTG

Appendix C : Troubleshooting

C.1 Library Preparation

1. Mis-handling of eBLT

- A. Beads are sensitive to drying, visually the analyst might see that certain samples do not resuspend as well on shake steps if beads have been overly dried. If bead clumping is observed, analyst should still continue. However, if library prep yield is low or no sample is detected this may be the cause.
- B. Beads should not be heated or frozen.
- C. Beads stored on their side, may dry before ever being used.
- D. If analyst is doing multiple preps, no more than 2 columns should be done at a time as this will cause drying during steps when liquid is removed.



E. Beads must be homogenous before use.

2. ST2 precipitation

- A. ST2 must be stored at room temperature, colder temperatures may cause precipitation.
- B. If ST2 has precipitates and is still used, loss of yield may be seen.

3. SPB cleanup

- A. If double SPB cleanup step is performed incorrectly, this may result in loss of sample or incorrect fragment length.

4. DNA input

- A. Ensure that DNA input is of expected quality and concentration. If library prep reaction quality is not as expected, input DNA should be tested using Qubit, to ensure expected concentration is found.

5. Indices

- A. Indices may be freeze/thawed up to 6 times (see *Cipher 24 UDI Pre-Verification Report*, 1000000043872), however different index pairs may be affected differently.
- B. Use a new index plate or new set of indices if Library Prep Yields are low. See *Titanium Verification Protocol IC Report*, 1000000063709 for more information on index performance.

6. Library Prep Yield

- A. If one or more samples fail Qubit quant due to reading being below detectable limit, running another Qubit on samples may be advisable prior to repeating Library Prep. Follow *Procedure, Out of Specification Procedure*, 15026737.
- B. If Library Prep Yield is low, samples may be run on a Bio Analyzer, desired fragment length is 350 bp, but may be between 275 bp and 385 bp. Fragment lengths outside of this range may impact yield and final results.

C.2 Enrichment

1. SPB cleanup (Library Prep)

- A. If double SPB cleanup step is performed incorrectly, and incorrect fragment size is achieved, this may impact enrichment yields.

2. Pooling

- A. If samples are pooled incorrectly, starting yield may be lower than expected leading to final enrichment yield being lower.
- B. Pooling incorrect amounts can lead to uneven representation in final data. If Library Prep Yields vary widely between samples, they may need to be pooled by concentration and not volume.

3. NHB1/NHB2 and EHB2 precipitation

- A. NHB1/NHB2 and EHB2 are prone to precipitation, ensure that NHB1/NHB2 is heated to 50°C before use and EHB2 is heated to room temperature. Vortex and look carefully for precipitation.

4. Capture and Wash steps

- A. Temperatures are critical, ensure that heat block is at appropriate temperature.
- B. Do not leave plate off of heat block or magnet for extended periods of time.
- C. Heating wash buffer aids in keeping the sample at the appropriate temperature.

5. Bio Analyzer

- A. If Library Prep Yield is low, samples may be run on a Bio Analyzer, desired fragment length is 350 bp, but may be between 275 bp and 385 bp. Fragment lengths outside of this range may impact yield and final results.

C.3 Data Analysis

1. Analysis does not start

- A. Samplesheet must be saved as a .csv file and must contain "TiSMBanalysis" for Project.
- B. If sequencing was started to obtain final results, sequencing must have completed successfully and have an RTAComplete file.

2. Analysis cannot complete successfully

- A. Check correct identity of indices and that the correct samples were removed from sample sheet. If incorrect indices were listed or a sample that was not a part of the final pool is listed, analysis will not be able to complete.
- B. Ensure that indexing in sequencer set up was set to 10 and not 8.
- C. Check that sample sheet is correctly filled out:
 - 1) Library Prep and Enrichment Yield values are present.
 - 2) TiSMBanalysis is listed as "Project"
 - 3) There are no spaces, commas, quotes, or other unexpected characters in Library and Enrichment reagent sections.

3. No email received for Start/Completion

- A. It is possible that emails can become delayed. Analysis may be running successfully or complete successfully without receiving an email. To check on analysis, navigate to run folder, if analysis has started a TiSMBanalysis. Started flag file will be present. If analysis is completed a TiSMBanalysis. Complete flag file will be present, all data along with PDF report will be in Analysis folder.



Title: WI, Nextera Flex for Enrichment SMB QC Assay
1000000103802, 00
Page 34 of 34

- B. Email not present, if email is not entered next to "Investigator", analysis will still launch but no emails will be received.

Release History

Version	ER #	Originator	Description of Change
00	1036920	Cody Thayer	Initial Release