General Protocol for amplicon library prep

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Viral RNA Isolation using the Maxwell 48

[description of samples]

Purpose

- Isolate viral RNA (vRNA) from samples using the Maxwell 48 instrument

Sample prep

1. Set heat block to 56C.
2. Perform Maxwell UV sanitation if it has not been done before.
3. Spin samples for 10,000g (rpg) for 5 minutes at 4C to pellet lung homogenate debris.
4. Prepare lysis solution by mixing proteinase K & lysis buffer according to the table on the right
5. Label a 1.5 ml microcentrifuge tube per sample + 1 negative control.
6. Aliquot 330µl **lysis solution** to each of the labeled microcentrifuge tubes.
7. Once samples are completely thawed, **vortex** and **spin** briefly to remove sample from lid of the tube.
8. Add 300µl **sample** to the appropriate micro centrifuge tube with lysis solution.
9. Add 300µl of **water** to the negative control.
10. Mix samples by **vortexing** and spin down briefly.
11. **Incubate** samples on 56C heat block for 10 minutes.

Sample tray prep: (during the 10 minute incubation)

1. **Change gloves**.
2. Turn on Maxwell and retrieve tray.
3. Visually inspect sample cartridges from viral nucleic acid purification kit to make sure volumes are correct and place 1 cartridge into the tray per sample.
4. Carefully peel the seal off of each sample cartridge.
5. Place an LEV plunger into well #8 of each cartridge (closest to you).
6. Place a **labeled** 0.5mL elution tube in front of each cartridge.
7. Add 50µl nuclease-free water (in the kit) to the bottom of each elution tube.
8. Following the 10 minute incubation, briefly spin down the samples.
9. Transfer all of the sample lysate to the well #1 of the cartridge (furthest from you).

Starting the run on the Maxwell

1. Select the viral program on the Maxwell and follow the screen instructions.
2. Place the sample onto the platform on the Maxwell correctly into place.
3. Press the Run/Stop button to begin the run. Close the door.
4. Transfer samples to 1.5ml espies for longer term storage or further use.

Reagent	Total per Rxn µl	# Rxns µl (+10%) 25
Lysis Buffer	300	8250.0
Proteinase K	30	825.0
Master Mix	**330**	**9075.0**

Notes:

This step is done in a BL3 facility

We include an extra 10% of reagents in each master mix to account for pipetting errors.

[initials] [date] Exp #

DNAse Treatment

[description of samples]

Protocol adapted from User Guide for TURBO DNA-free Kit (Cat No. AM1907 Ambion by Life Technologies)

Purpose

- Remove any DNA from viral RNA samples using TURBO DNA-free Kit in preparation for RT-PCR and library prep.

Protocol

1. Clean the hood with 10% bleach; Place a bleached tube rack and discard bucket in the hood.
2. Prepare a labeled PCR tube for each sample
3. Verify that the thermocycler is available
4. Create a master mix using the components in the chart on the right.
5. Add 3 uL master mix for each tube to be used.
    1. Each DNAse treated vRNA sample will be used for HA rep 1 and PA rep 1. Remaining vRNA will be used later (Store at -80C)
    2. Add **20 µl vRNA sample** to each tube or well and mix gently by pipetting.
6. Incubate tubes at 37C for 25 mins on the thermocycler. (can increase/decrease between 20-30 mins)
7. Re-suspend DNase Inactivation Reagent by flicking or vortexing.
8. **Add 2 uL** (0.1 volume) per 20 uL RNA of **DNase Inactivation Reagent** to each tube or well and mix well.
    1. Note: Always use at least 2 uL DNase Inactivation Reagent even if less than 0.1 volume
    2. If DNase Inactivation Reagent becomes difficult to re-suspend after multiple uses, add volume of nuclease-free water (in kit) equal to 20-25% of the volume of the remaining DNase Inactivation reagent.
    3. Vortex thoroughly
9. Incubate at room temperature (22-26C) for **5 minutes.** Flick tube 2-3 times during incubation to re-disperse DNase inactivation reagent.
10. Label **two** 1.5ml tubes for each sample and transfer the contents from the PCR tube to one of the 1.5mL tubes
11. Centrifuge tubes 1.5 mins at 10,000 X g (rpf).
12. Transfer clear supernatant containing RNA to the second (fresh) 1.5ml tube. **Be very careful not to disturb the DNase Inactivation reagent (pellet)**.
13. **Label a box with your initials and a "DNAse treated" label and store the samples at -80C**
14. Note: For use of RNA in QRT-PCR, treated RNA should comprise ~20% and no more than 40% of the total QRTExp

Reagent	Stock Conc.	Total per Rxn µl	# Rxns µl (+10%)
			25
10x TURBO DNAse Buffer	10x	2	55.0
TURBO DNAse	10µl	1	27.5
Master Mix		**3**	**82.5**

Notes:

- Protocol is designed to remove up to 50 ug DNA/mL RNA.
- Recommended reaction size is 10-100 uL. Typical reaction size is 50 uL
- This protocol is designed to treat samples with <=200 ug nucleic acid/mL. For >200 ug nucleic acid or >2 ug DNA /50 uL RNA, see TURBO DNA-free User Guide.
- Treatment may influence accurate RNA quantification for samples with RNA concentration below 10 ng/uL.

[initials] [date] Exp #

Reverse transcription, first strand synthesis

[description of samples]

Purpose

- Convert vRNA to cDNA

Protocol (VILO_Reverse_Transcription)

1. Label PCR tubes (2 for each sample - HA and PA in separate reactions)
2. Add RNAseout to DEPC-treated water (**10ul to 1mL** or equivalent)
3. Add the following components to an empty RNAse-free tubes on ice **separately (one for HA and the other for PA, each with their respective primers)**

Reagent	Amount/tube (µl)	Master Mix(# samples + 10%) 1RT 10	Master Mix(# samples + 10%) 3RT 30
SSIV VILO Master Mix	4	44	132
Template RNA	3	Add separately	Add separately
Primer	1	11	33
DECP-water (+ 1:10000 RNAase)	12	132	396
Total Volume	Should be total of 17µl of MM per tube before adding the template RNA (total of 20µl)		

5. Mix and briefly centrifuge.
6. Incubate at 25C for 10 minutes. (Anneal primers).
7. Incubate at 50C for 30 minutes. (Reverse transcribe RNA to cDNA).
8. Incubate at 85C for 5 minutes. (Inactivate the SSIV VILO enzyme).
9. To remove RNA, add 1ul of RNAseH, incubate at 37C for 20'. (Remove the RNA).
10. Use cDNA for PCR amplification right away or store at -20C.

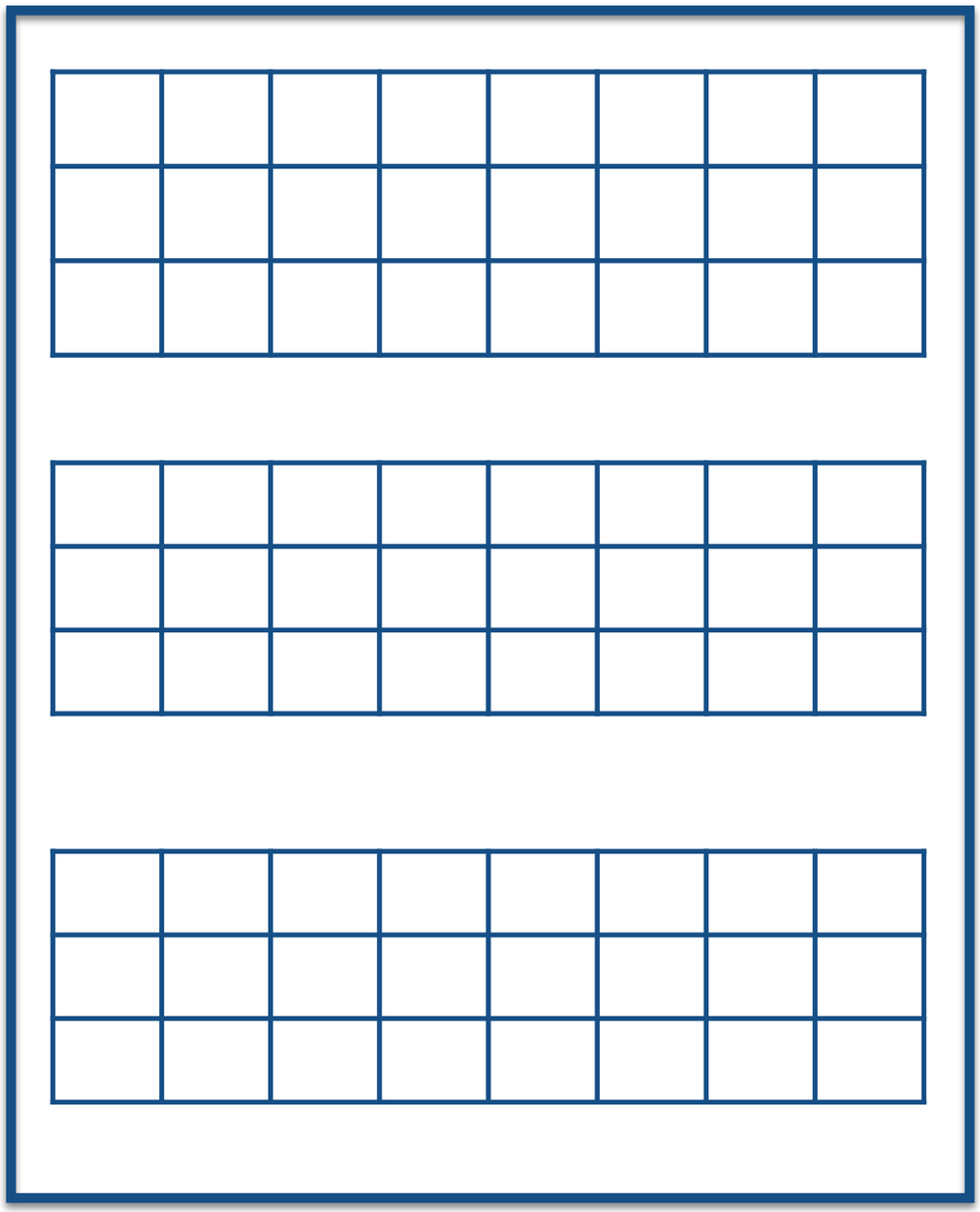
Notes:

Depending on the protocol and samples, we can either do 1-RT reaction per sample or 3-RT reactions per sample.

If doing 3-RT reactions, follow those 3-RT reactions for the PCR1 step, and then combine them before PCR2 (last column).

[initials] [date] Exp #

Labeling guide for RT tubes



Each square corresponds to an individual PCR tube. Each line corresponds to a PCR strip

[initials] [date] Exp #

PCR1 - Adding partial adapters

[description of samples]

Purpose

- Amplify cDNA with segment-specific primers to add partial adapters.

Protocol

1. In the clean room, clean the hood with 10% bleach; place a bleached tube rack and discard bucket in the hood.
2. Get ice, racks and label clean PCR tubes
3. Complete thaw and vortex of Phusion reagents (except the enzyme, which should stay on ice) before use.
4. Label PCR tubes prior to making master mixes.
5. Re-suspend lyophilized primers if needed (multiply the nanomolarity x10 and add that amount in clean water - then aliquot from the resuspended stock and make a 1:10 dilution to use it for the MM).
    1. Example: primer is 29.0nmol - add 290ul of clean water and resuspend to have a **100um stock**. Aliquot 50ul to a clean tube and add 450ul of clean water to have a 10um stock)
6. Prepare master mixes following the table below
7. Aliquot master mix first into each PCR tube
8. Add template cDNA to each tube and pipette up and down to mix.
9. Start thermo-cycler according to the table on the right.

Reagent	Amount/tube (µl)	Master Mix(# samples + 10%) x1 15	Master Mix(# samples + 10%) x3 45
2x Phusion MM	10	165	495
Template cDNA	2	Add separately	Add separately
Forward primer (noUMI)	1	16.5	49.5
Reverse primer	1	16.5	49.5
Water	6	99	297
Total Volume	Should be total of 18µl of MM per tube before adding the template cDNA (total of 20µl)		

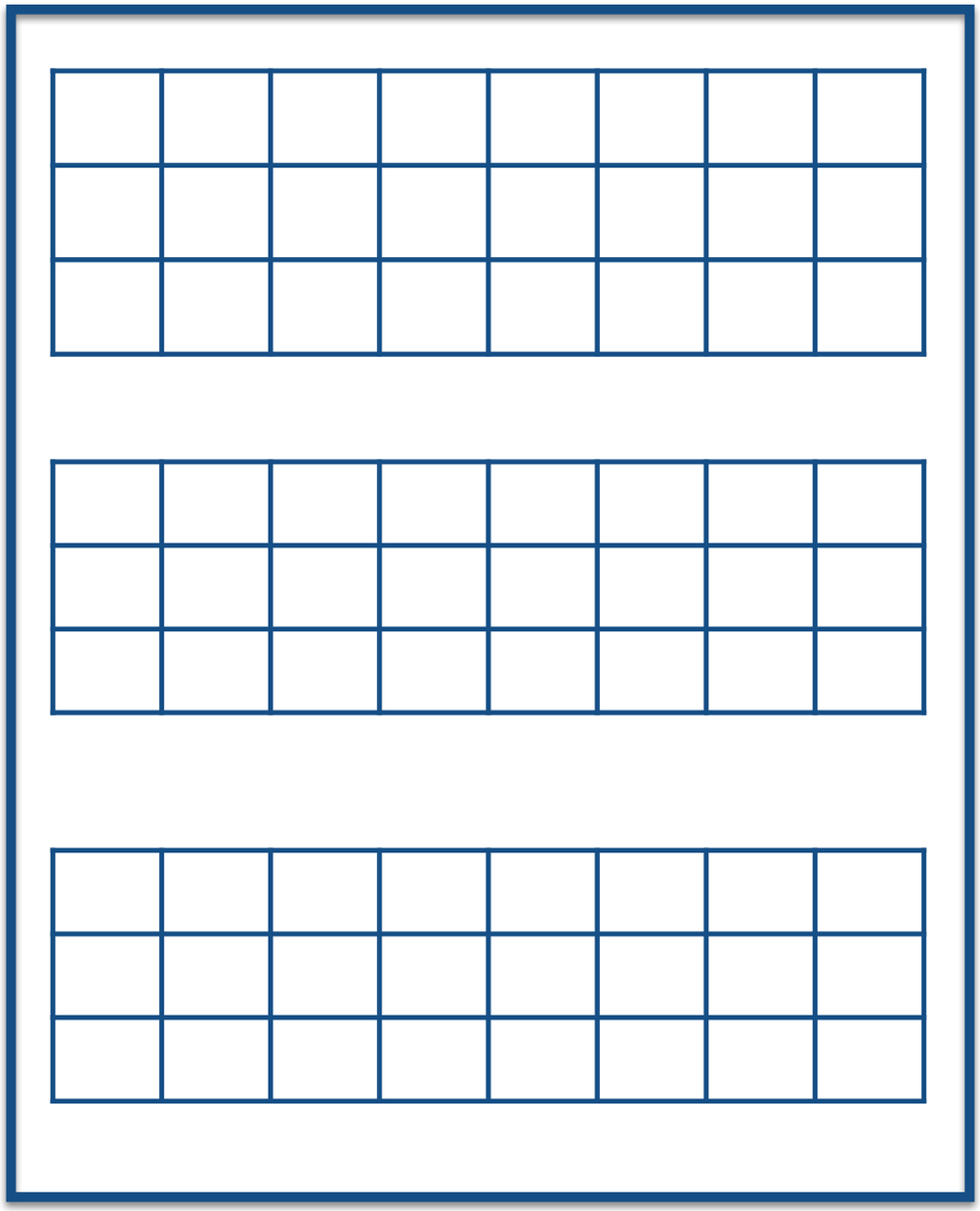
Gene Segment	FWD primer name	REV primer name	Expected Amplicon length
HA	HA_noUMI_(PCR1)	HA_rev3_PCR1_v3	284
PA	PA_noUMI_(PCR1)	PA_rev3_PCR1_v3	350

Thermo-cycler settings for HA segment			
HA		Temp C	Time
Initial denaturation		98	30s
25 cycles	Denature	98	10s
	Anneal	59	30s
	Extend	72	30s
Final extension		72	5min
Hold		4	∞

Thermo-cycler settings for HA segment			
PA		Temp C	Time
Initial denaturation		98	30s
25 cycles	Denature	98	10s
	Anneal	63	30s
	Extend	72	30s
Final extension		72	5min
Hold		4	∞

[initials] [date] Exp #

Labeling guide for PCR1 tubes



Each square corresponds to an individual PCR tube. Each line corresponds to a PCR strip

[initials] [date] Exp #

PCR1 product purification

[description of samples]

Purpose

- Clean amplified DNA to get rid of RT-PCR reagents + primers using the AMPureXP PCR Cleanup Kit

PCR Purification Protocol using AMPureXP PCR Cleanup Kit

1. Clean bench with bleach and 70% ethanol.
2. Label **two separate sets** of PCR tubes per sample and get a magnetic tube rack
3. Prepare fresh 80% Ethanol
4. Combine 20µl from all three PCR replicates into one of the labeled PCR tube set = ~60µl.
5. Beads should be room temp (ish) before use. Resuspend the bottle and transfer ~1ml to a 1.5ml tube. Vortex the 1 ml aliquot and use this aliquot to add it to your samples. keep resuspending them before use.
6. **Add 48µl of the resuspended beads to the 60µl of (0.8x) DNA.**
7. Mix the AMPureXP and samples thoroughly. Let the tube incubate at room temperature for **5 minutes** before proceeding to the next step (incubate off of the magnetic rack).
8. Place the tube onto the magnetic tube rack for 5 minutes to separate the beads from solution.
9. **Slowly** aspirate the **cleared solution from the tube and discard.** This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.
10. Dispense **200 µl** of freshly made **80% ethanol** into the tube and incubate for **30 seconds** at room temperature. Aspirate out the ethanol and discard. Repeat for a total of **TWO washes**. It is important to perform these steps with the **tube situated on the rack.** Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well with a P20 it may contain residual contaminants.
11. Let the reaction tube air-dry for a few minutes on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery.
12. **Remove** tube from magnetic rack and resuspend beads in **12µl DNase-free water** (eluate) by pipetting up and down. Place tube back on the magnetic rack until the solution is clear. Pipette **10ul eluant** from the tube to the second labeled PCR tube set while the sample is still situated on the magnetic tube rack .

[initials] [date] Exp #

PCR2 - Adding sequencing adapters and indices

[description of samples]

Purpose

- Amplify DNA to add remaining adapters and indices

Protocol

1. Clean the bench with 10% bleach & 70% ethanol; Place a bleached tube rack and discard bucket in the bench.
2. Get ice, racks and clean PCR tubes.
3. Complete thaw and Phusion, primers and samples before use.
4. Label PCR tubes prior to making master mixes (see next slide). **10 samples + 2 negative controls = 12 tubes (2 strips)**
5. Prepare master mix over ice if possible. Prepare one master mix for the two segments (Table below).
6. Aliquot master mix, corresponding index and then aliquot DNA from PCR1.
7. After adding DNA to each tube, pipette up and down to mix.
8. Load in the thermocycler according to the table on the right.

Primer	Name
Rev	Uni_fwd_addseqadaptors_PCR2rev
Fwd	Uni_rev_index*_addseqadaptors_PCR2fwd SEE TABLE ON THE RIGHT

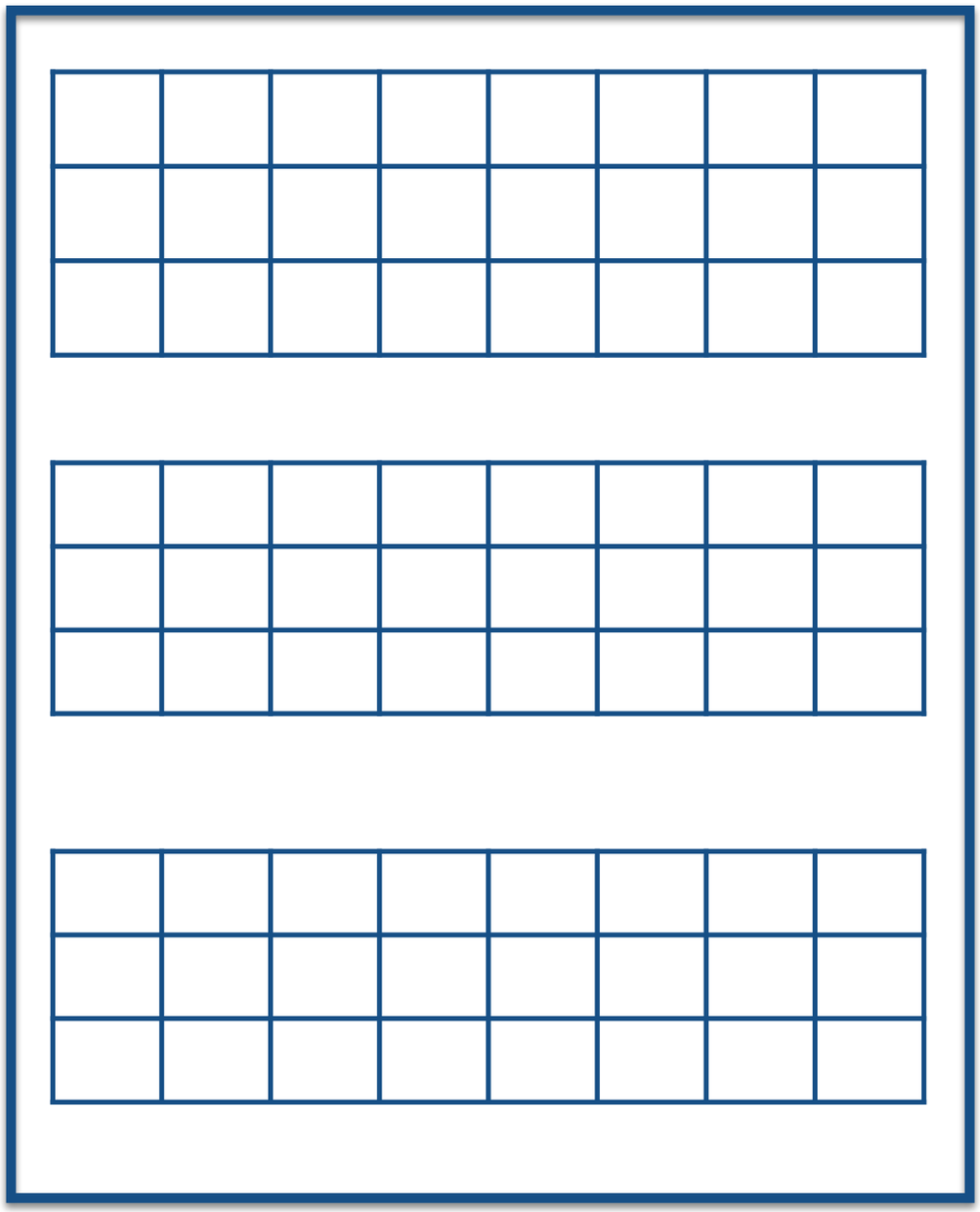
Thermo-cycler settings for HA segment			
HA / PA		Temp C	Time
Initial denaturation		98	30s
30 cycles	Denature	98	10s
	Anneal	72	30s
	Extend	72	30s
Final extension		72	5min
Hold		4	∞

Reagent	Amount/tube (µl)	Master Mix(# samples + 10%) x1 14
2x Phusion MM	10	154
Template cDNA	2	Add separately
Forward primer (indexed)	1	Add separately
Reverse primer	1	15.4
Water	6	92.4
Total Volume	Should be total of 17µl of MM per tube before adding the template cDNA (total of 20µl)	

Sample	index
	1
	2
	3
	4
	5
	6
	7
	8
	9
	10
	11
	12
	13
	14
	15
	16
	18
	19
	20
	21
	22
	23
	25
	27

[initials] [date] Exp #

Labeling guide for PCR2 tubes



Each square corresponds to an individual PCR tube. Each line corresponds to a PCR strip

[initials] [date] Exp #

Extraction of PCR2 products from Gel

[description of samples]

Purpose

- Purify the PCR2 products by running a 1% agarose gel
- Extract DNA amplicons (bands) from gel using the QIAquick Gel Extraction Kit (QIAgen)

Preparation and loading of the Gel

1. Clean and assemble the tray without the comb.
2. For one 1% agarose gel, combine 90mL of 1XTAE buffer with 0.9g of agarose and heat using the microwave, swirling, and until the solution is clear.
3. Add 9µl of SYBR Safe reagent (located in a white cylinder on top of Kat's bench) and mix well.
4. Pour into the gel tray and carefully add the **two** combs. Each comb consists of 14 sample spots. Cover with plastic wrap and let it solidify.
5. While the gels solidify, add 4µl of 5x gel dye (located in the white cylinder) and pipette up and down to mix. You should have a total of ~24µl of sample+dye.
6. When the gel is solid, remove the combs and fill with fresh TAE buffer.
7. Add 10µl of the ladders (1kb and 100bp) and 24µl of samples following the table on the right. (1kb ladder is located on the white cylinder and the 100bp on the TCF fridge) and **run the gel at 110V for 35 minutes**

DNA Extraction

1. Label two 1.5ml tubes per sample. Also label a 2mL QIAquick collection tube per sample. Start heat block at 50C and get a waste bucket.
2. View gels on UV light. **Photograph the gel for our records with a tape containing the information of the exp.**
3. Excise bands (wear eye protection), put them on their corresponding clean pre-labeled 1.5ml tubes. **Photograph gel after removing the bands with a tape containing the information of the exp.**
4. **Record weigh on next slide** (max weight should be 400mg).
5. Add **3 volumes of Buffer QG per 1 gel volume** to the band and incubate for **10 minutes at 50C** or until gel is completely dissolved. In between the 10 minutes, **vortex** 2-3 times to help the gel dissolve.
6. Add **1 gel volume of isopropanol** to the sample and mix
7. Place a QIAquick spin column on the QIAquick collection tube. Transfer up to **800µl of the dissolved gel** and spin for **1 minute at 13,000 rpm**. If more than 800µl, load and spin again. Discard flow-through.
8. Add **500µl of Buffer QG** to the column and centrifuge for 1 minute at 13,000 rpm. Discard flow-through.
9. Add **750µl of Buffer PE (make sure it has ethanol added)** to the column, **incubate** for 2-5 minutes at room temperature and spin for 1 minute at 13,000 rpm. Discard flow though. **Repeat spin a second time to remove excess of the PE buffer.**
10. Place the QIAquick column on the pre-labeled clean 1.5ml tube and add **12µl of clean elution buffer (of water)** and incubate for 3-4 minutes at room temperature. Spin for 1 minute at 13,000 rpm and save your eluted sample.

GEL 1	
Sample	well
Ladder (1K)	1-1
Ladder(100bp)	1-2
	1-3
	1-4
	1-5
	1-6
	1-7
	1-8
	1-9
	1-10
	1-11
	1-12
	1-13
	1-14
Ladder (1K)	2-1
Ladder(100bp)	2-2
	2-3
	2-4
	2-5
	2-6
	2-7
	2-8
	2-9
	2-10
	2-11
	2-12
	2-13
	2-14

GEL 2	
Sample	well
Ladder (1K)	1-1
Ladder(100bp)	1-2
	1-3
	1-4
	1-5
	1-6
	1-7
	1-8
	1-9
	1-10
	1-11
	1-12
	1-13
	1-14
Ladder (1K)	2-1
Ladder(100bp)	2-2
	2-3
	2-4
	2-5
	2-6
	2-7
	2-8
	2-9
	2-10
	2-11
	2-12
	2-13
	2-14

[initials] [date] Exp #

Extraction of PCR2 products from Gel

[description of samples]

Sample	Index	Weight/Isoprop.	QC	Qubit (ng/µl)
	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	0	0	0
	7	0	0	0
	8	0	0	0
	9	0	0	0
	10	0	0	0
	11	0	0	0
	12	0	0	0
	13	0	0	0
	14	0	0	0
	15	0	0	0
	16	0	0	0
	18	0	0	0
	19	0	0	0
	20	0	0	0
	21	0	0	0
	22	0	0	0
	23	0	0	0
	25	0	0	0
	27	0	0	0

[initials] [date] Exp #

Post **PCR2**- Gel Images

Gene Segment:

Purpose
- Confirmation of amplified DNA (PCR1 product)

Gene segment	Expected length
HA	~355
PA	~420

Before extraction

After extraction

Notes:

[initials] [date] Exp #

Quality Control

[description of samples]

Qubit Buffers

Purpose

- Quality control of the final products (purified DNA from PCR2) using Qubit Buffers for measuring DNA concentration

Preparation

1. Label one Qubit tube per sample and two standards as S1 and S2.
2. Add **190µl of Qubit Buffer** to the two standard tubes.
3. Add **199µl of Qubit Buffer** to the sample tubes
4. Add **10µl of the two standards** to their respective tubes (S1 and S2).
5. Ad 1**µl of each purified DNA** to their respective tubes (with Qubit buffer).
6. Locate the instrument and select hsDNA setting
7. **Vortex each sample** before readings.
8. Start with S1 and S2 (follow directions on the instrument).
9. Make sure the **volume is set to 1µl** and that the reading is in **ng/µl.**
10. **Record every value** of the fluorimeter (table on the right).

Notes:

Sample	Index	Qubit (ng/µl)	Dilution (if needed)	
			Water (µl)	Sample (µl)
	1	0	-1	1
	2	0	-1	1
	3	0	-1	1
	4	0	-1	1
	5	0	-1	1
	6	0	-1	1
	7	0	-1	1
	8	0	-1	1
	9	0	-1	1
	10	0	-1	1
	11	0	-1	1
	12	0	-1	1
	13	0	-1	1
	14	0	-1	1
	15	0	-1	1
	16	0	-1	1
	18	0	-1	1
	19	0	-1	1
	20	0	-1	1
	21	0	-1	1
	22	0	-1	1
	23	0	-1	1
	25	0	-1	1
	27	0	-1	1

[initials] [date] Exp #

Bioanalyzer: Preparing gel dye

[description of samples]

Instrument**: Agilent High Sensitivity DNA Assay / 2100 Bioanalyzer instrument**

Purpose

- DNA analysis/quality control: High-resolution separation of bands, automated sizing, quantification and purity analysis of DNA prior to sequencing

Preparing Gel-Dye mix

1. High Sensitivity DNA reagents are stored in the TCF fridge. **BLUE** capped reagent is the High Sensitivity DNA dye and the **RED** capped is the High Sensitivity DNA matrix. **Allow both to equilibrate to room temperature for ~30 minutes**.
2. Vortex the **BLUE** dye for 10 seconds and spin down. Pipette 15µl of the **BLUE** capped dye into to the **RED** capped matrix tube. Store the blue dye on the dark immediately.
3. Vortex the **BLUE-RED** dye-matrix mix for 10 seconds and visually inspect for proper mixing of both reagents.
4. Transfer the **BLUE-RED** dye-matrix to a spin filter.
5. Place the spin filter in a microcentrifuge and spin for 10 minutes at 2240 g +/- 20% (6000 rpm if using Eppendorf centrifuge)
6. Label the microcentrifuge tube with the date of preparation and the reagent name. The dye-matrix reagent can be stored in the dark and used for up to 6 weeks and it is sufficient for up to 5 chips. Store at 4C when not in use for more than 1 hour.

Notes:

[initials] [date] Exp #

Bioanalyzer: Loading Chip

[description of samples]

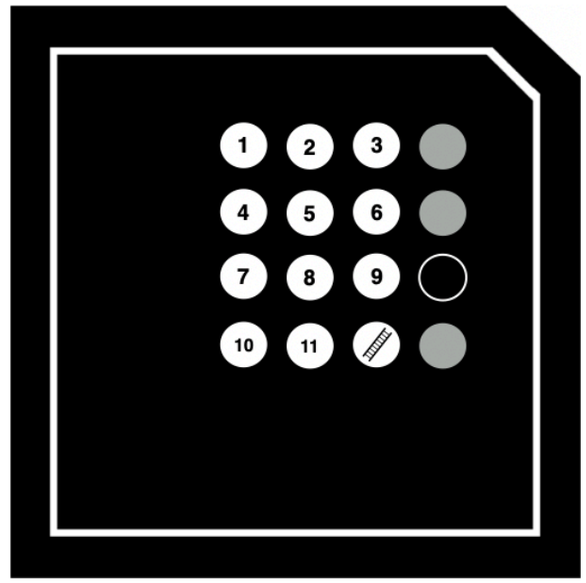
Instrument**: Agilent High Sensitivity DNA Assay / 2100 Bioanalyzer instrument**

Purpose

- DNA analysis/quality control: High-resolution separation of bands, automated sizing, quantification and purity analysis of DNA prior to sequencing

Loading the Chip

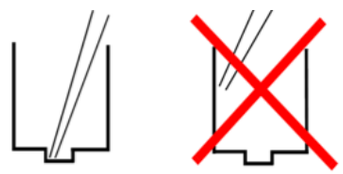
1. Equilibrate the gel dye to room temperature and take a new DNA chip, load it into the priming station with the plunger set at 1ml and set a timer for 60 seconds.
2. Load 9µl of the gel dye into the well marked with a **G** (black circle on the right).
3. After loading the gel dye, close the priming station carefully until you hear the click cause by the gray clip.
4. Press the plunger all the way down until it is held by the clip and immediately start the 60 second countdown.
5. After the 60 second ends, release the plunger using the same clip and inspect that it moves back to at least 0.3ml.
6. Wait for 5 seconds and pull the plunger back to 1ml. Open the priming station.
7. Add 9µl to the remaining wells marked with **G** (gray wells on the right)
8. Add 5µl of the green-capped marker to each of the remaining 12 wells (shown as white circles on the right)
9. Vortex the yellow-capped ladder for a few seconds and pipette 1µl of the ladder to the corresponding well (well #12, shown here with a black ladder)
10. Add 1µl of DNA samples to the wells labeled 1-11 (see next slide)



Notes:
Using gel loading dye made in January

https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf

For some wells, we combined 2 samples by adding 0.5µl of each sample.

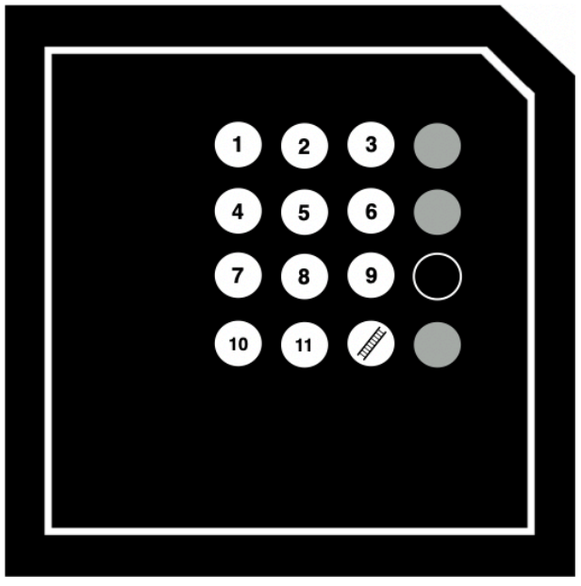


[initials] [date] Exp #

Bioanalyzer: Chip Assignments

[description of samples]

Sample	Index	Qubit (ng/µl)	Amount for bioanalyzer	Assigned well on Chip	Avg BP
	1	0	1µl		
	2	0	1µl		
	3	0	1µl		
	4	0	1µl		
	5	0	1µl		
	6	0	1µl		
	7	0	1µl		
	8	0	1µl		
	9	0	1µl		
	10	0	1µl		
	11	0	1µl		
	12	0	1µl		
	13	0	1µl		
	14	0	1µl		
	15	0	1µl		
	16	0	1µl		
	18	0	1µl		
	19	0	1µl		
	20	0	1µl		
	21	0	1µl		
	22	0	1µl		
	23	0	1µl		
	25	0	1µl		
	27	0	1µl		



[initials] [date] Exp #

Bioanalyzer: Traces

[description of samples]

[initials] [date] Exp #

Sample Summary (print and include in box for storage)

[description of samples]

Sample	Index	Dilution (if included)			
		Qubit (ng/µl)	Water (µl)	Sample (µl)	BA length
	1	0	-1	1	
	2	0	-1	1	
	3	0	-1	1	
	4	0	-1	1	
	5	0	-1	1	
	6	0	-1	1	
	7	0	-1	1	
	8	0	-1	1	
	9	0	-1	1	
	10	0	-1	1	
	11	0	-1	1	
	12	0	-1	1	
	13	0	-1	1	
	14	0	-1	1	
	15	0	-1	1	
	16	0	-1	1	
	18	0	-1	1	
	19	0	-1	1	
	20	0	-1	1	
	21	0	-1	1	
	22	0	-1	1	
	23	0	-1	1	
	25	0	-1	1	
	27	0	-1	1	

[initials] [date] Exp #

Illumina MiSeq protocol (print and include in box)

[description of samples]

Run Reference Numbers:	
Library #	
Experiment #	
Run #	
FLow Cell ID	

Instrument**: Illumina MiSeq (v2 Kit 500 cycles)**

Purpose

- DNA amplicon sequencing (paired-end, short-read)

Protocol

1. Thaw HT1 on ice
2. Place cartridge on water (room temperature). Begin thawing cartridge before denaturing and diluting libraries
3. Prepare fresh 0.2N NAOH (pipette accurately)
    1. 800ul DEPC water
    2. 200ul 1.0N NaOH
    3. 1mL of **0.2N NaOH**
    4. **Invert the tube to mix**

	Library	**PhiX control**
Create 4nM library	**Use sample sheet (excel)**	**2ul of 20nM PhiX control + 3ul of 10mM Tris-HCl, pH 8.5, with 0.1% tween 20**
Denature library with 0.2N NaOH Vortex briefly and centrifuge at 280xg for 1 minute Incubate at room temperature for 5 minutes. Should have 10ul in both libraries	**5ul of 4nM library + 5ul 0.2N NaOH**	**5ul 4nM PhiX library + 5ul 0.2N NaOH**
Create 1mL of 20pM library Same tube after incubation with NaOH	**Add 990ul cold HT1 to 10ul denatured library**	**Add 990ul cold HT1 to 10ul denatured library**
Create 600ul of 8pM library Transfer 20pM aliquot to a new tube then add cold HT1 Invert to mix and then pulse centrifuge	**240ul 20pM Library pool + 360ul cold HT1**	**240ul 20pM PhiX + 360ul cold HT1**
Final Library **Total of 600ul (8pM pool + 30% PhiX)** Transfer amount from each library to a new tube	**420ul 8pM library**	**180ul 8pM PhiX**
	600ul 8pM library + 30% PhiX control Load this amount on the sample well of the Illumina cartridge	

[initials] [date] Exp #