Asuragen®

QuantideX®

NGS RNA Lung Cancer Kit

Protocol Guide

49604

For Research Use Only. *Not for use in diagnostic procedures.*
REF **49602, 49603**
Σ **48 - 192**

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Related Protocols

- PC-0249, QuantideX® NGS Reporter User Guide, available at http://quantidex.asuragen.com
- MiSeq® System User Guide, available at http://support.illumina.com/
- Use these protocols in conjunction with the following protocol to successfully execute the QuantideX® NGS RNA Lung Cancer Kit library prep workflow.

Contact Asuragen Technical Support if any problems are encountered when using this product:
support@asuragen.com and at +1.512.682.5200

QuantideX® NGS RNA Lung Cancer Kit Protocol Overview

Reverse Transcription (RT)

- Kit: QuantideX® NGS RNA Assay (Lung) [Box 1]
  - 4 µL QuantideX® RT Buffer
  - 1 µL QuantideX® RT Enzyme Mix
  - 5 µL RNA/TNA sample, NTC or RNA Positive Fusion Ctrl (provided)

Temp	Time
42°C	45 min
93°C	10 min
4°C	hold

RNA Assay

- Kit: QuantideX® NGS RNA Assay (Lung) [Box 1]
  - 5 µL 2X qPCR Master Mix
  - 2.5 µL Primer/Probe Mix (Lung)
  - 0.5 µL 20X ROX (if needed)
  - 2 µL RT product or qPCR Standards 1 - 4 (Lung)

Temp	Time	Cycling
95°C	5 min	-
95°C	5 sec	40 cycles
60°C	60 sec	

qPCR using FAM channel

Gene-Specific PCR

- Kit: QuantideX® NGS RNA Lung Panel [Box 2]
  - 5 µL 2X Amplification Master Mix
  - 1 µL RNA Lung Primer Mix
  - 4 µL RT product
  - (Suggested input ≥ 400 functional copies)

Temp	Time	Cycling
95°C	5 min	-
95°C	15 sec	2 cycles
60°C	4 min	
95°C	15 sec	23 cycles
72°C	4 min	
72°C	10 min	-
4°C	hold	-

Tagging PCR

- Kits: QuantideX® NGS RNA Lung Panel [Box 2] & QuantideX® Codes - Set A/B/C/D [Box 3]
  - 7.5 µL 2X Index Master Mix
  - 5.5 µL Index Code (AIL001-AIL192)
  - 2 µL Gene-Specific PCR product

Temp	Time	Cycling
95°C	5 min	-
95°C	30 sec	10 cycles
55°C	30 sec	
72°C	60 sec	
72°C	10 min	-
4°C	hold	-

Library Purification

- Kit: QuantideX® NGS Library Pure Prep [Box 4]
  - 11 µL Library Pure Prep Beads
  - 10 µL Tagging PCR product
  - 2 X 100 µL washes w/ EtOH-diluted Wash Buffer
  - Elute in 20 µL Elution Buffer

Mix, pellet beads on magnet, remove supernatant
Leave plate on magnet
Elute 5 min, recover pure products

Library Quantification

- Kit: QuantideX® NGS Library Quant [Box 5]
  - 5 µL 2X qPCR Master Mix
  - 2.5 µL LQ Primer/Probe Mix
  - 0.5 µL 20X ROX (if needed)
  - 2 µL Diluted purified product (diluted 10,000-fold in LQ Diluent) or LQ Standards 1 - 4

Temp	Time	Cycling
95°C	5 min	-
95°C	5 sec	35 cycles
60°C	60 sec	

qPCR using FAM channel

Pool, Denature, Dilute, Load

- Kits: QuantideX® NGS Sequencing Reagents [Box 6] & Illumina® MiSeq® Reagent Kit
  - 15 µL 2.5 nM normalized library pool from stock purified products
  - 3 µL 1 nM PhiX Control V3, 2 µL 1 N NaOH; denature for 5 min at room temp, snap cool on ice
  - Dilute 8 µL of the denatured library into 992 µL pre-chilled HT1 buffer
  - Dilute 4 µL Read 1, Index Read, & Read 2 Primers in 636 µL HT1 buffer individually, load MiSeq®

QuantideX® NGS RNA Lung Cancer Kit Components

Reference No.	Description	Cap color, if applicable	Vol/Rxns supported	Storage Temperature
49589	QuantideX® NGS RNA Assay (Lung) [Box 1]		48 Reactions	-15 to -30°C
145435	QuantideX® RT Enzyme Mix	green	48 µL	-15 to -30°C
145436	QuantideX® RT Buffer	green	192 µL	-15 to -30°C
145442	RNA Fusion Positive Ctrl (Lung)	clear	240 µL	-15 to -30°C
145437-40	qPCR Standards 1 - 4 (Lung)	white	32 µL each	-15 to -30°C
145444	2X qPCR Master Mix	yellow	360 µL x 2	-15 to -30°C
145443	Primer/Probe Mix (Lung)	blue	280 µL	-15 to -30°C
145441	20X ROX	amber	56 µL	2 to 8°C (after 1st use)
49588	QuantideX® NGS RNA Lung Panel [Box 2]		48 Reactions	-15 to -30°C
145348	2X Amplification Master Mix	green	240 µL	-15 to -30°C
150016	RNA Lung Primer Mix	green	48 µL	
145361	2X Index Master Mix	yellow	360 µL	
49553-49556	QuantideX® NGS Codes - Set A/B/C/D* [Box 3]		192 Reactions	-15 to -30°C
150004-150007	Index Codes (ILM) - Set A/B/C/D	rack of codes	22 µL	2 to 8°C (after 1st use)
49551	QuantideX® NGS Library Pure Prep [Box 4]		48 Reactions	2 to 8°C
145351	Library Pure Prep Beads	amber	530 µL	2 to 8°C
145352	Wash Buffer	bottle	2.5 mL	
145353	Elution Buffer	blue	1200 µL x 2	
49590	QuantideX® NGS Library Quant [Box 5]		48 Reactions	-15 to -30°C
145445	LQ Diluent	bottle	19.2 mL	2 to 8°C (after 1st use)
145444	2X qPCR Master Mix	yellow	360 µL x 2	-15 to -30°C
145447	LQ Primer/Probe Mix	violet	280 µL	-15 to -30°C
145441	20X ROX	amber	56 µL	2 to 8°C (after 1st use)
145449-52	LQ Standards 1 - 4	blue	32 µL	-15 to -30°C
49557	QuantideX® NGS Sequencing Reagents [Box 6]		8 Runs	2 to 8°C
145365	Sequencing Diluent	blue	1200 µL	2 to 8°C
150001	Read 1 Sequencing Primers	green	32 µL	
150002	Index Read Sequencing Primers	green	32 µL	
150003	Read 2 Sequencing Primers	green	32 µL	

*Only one of the four sets of Index Codes are included when ordering the QuantideX® NGS RNA Lung Cancer Kit. See Appendix 1 for more details regarding the QuantideX® NGS Codes.

Notes: Box numbers are included on box labels. Reactions supported assumes approximately using 15% overage when preparing reaction master mixes.

The QuantideX® NGS Reporter (49562), a bioinformatics solution for analyzing MiSeq output files, is included in the purchase of the kit. See the QuantideX® NGS Reporter User Guide (PC-0249), available at http://quantidex.asuragen.com/ for download.

Kit Configuration

Box 1: QuantideX® NGS RNA Assay (Lung)

- QuantideX® RT Buffer
- QuantideX® RT Enzyme Mix
- RNA Fusion Positive Control
- qPCR Standards 1-4 (Lung)
- 2X qPCR Master Mix
- Primer/Probe Mix (Lung)
- 20X ROX

Box 2: QuantideX® NGS RNA Lung Cancer Kit

- 2X Amplification Master Mix
- RNA Lung Primer Mix
- 2X Index Master Mix

Box 3: QuantideX® NGS Codes - Set A to D

Box 4: QuantideX® NGS Library Pure Prep

- Library Pure Prep Beads
- Elution Buffer
- Wash Buffer

Box 5: QuantideX® NGS Library Quant

- LQ Primer/Probe Mix
- 2X qPCR Master Mix
- LQ Standards 1-4
- LQ Diluent
- 20X ROX

Box 6: QuantideX® NGS Sequencing Reagents

- Read 1 Sequencing Primers
- Index Read Sequencing Primers
- Read 2 Sequencing Primers
- Sequencing Diluent

Required Materials Not Provided

Suggested part numbers have been provided for reagents and consumables, but equivalent products may be used. Consult the MiSeq System User Guide for additional materials required to use Illumina MiSeq instrument.

- Nuclease-free water
- 96-100% Ethanol
- 1 N Sodium Hydroxide
- PhiX Control v3 (Illumina 15017666)
- Round-bottom plates (Evergreen 290-8117-01R)
- Thermal cycler
- PCR plates and seals
- qPCR instrument†
- Optical plates and seals
- Magnetic plate stand (Ambion AM10027)
- Micronic tube decapper (Univo SR008, or equiv.)
- Illumina MiSeq Desktop Sequencer and MiSeq Reagent Kit

Optimal number of samples for each MiSeq Reagent Kit

Reagent Kit	Catalog#	Number of Reads ‡	Recommended Number of Samples with Kit*
MiSeq Reagent Kit v3, 600 cycles	MS-102-3003	25 million	8-48
MiSeq Reagent Kit v2, 500 cycles	MS-102-2003	15 million	4-24

† If an ABI 7500 (Fast or Standard) or Roche 480 qPCR instrument is unavailable for qPCR, consult Asuragen Tech Support for guidance on use of other platforms.

‡ This information is provided by Illumina. For more information, consult Illumina for MiSeq® System Specification.

* The number of samples for each MiSeq reagent kit is recommended to achieve the optimized QuantideX® NGS RNA Lung data output. Consult Asuragen Tech Support for further assistance.

Optional Equipment Not Provided

- Flatbed or handheld scanner with Data Matrix 2D barcode reading capabilities

Intended Use

The QuantideX® NGS RNA Lung Cancer Kit is intended for the detection of clinically-relevant content for RNA targets common to lung cancer including both fusions as well as mRNA expression profiling for relevant genes from RNA or TNA purified from human tissue, FFPE or cell-lines. The kit covers 107 specific RNA fusions (shown below), MET exon 14 skipping, 3'-5' imbalance ratios for 5 RNA expression markers (See Appendix 3) involved in oncogenic translocations, and 23 expression markers that are semi-quantitatively evaluated against a set of low variation reference genes. The kit supports multiplex next-generation sequencing analysis with an Illumina® MiSeq® instrument. The kit includes software (QuantideX® NGS Reporter, 49562) that analyzes MiSeq® data files for the identification of fusion targets using a locally integrated bioinformatic pipeline and companion data visualization tools.

Fusion Targets

3' Gene Partner	5' Gene Partner	COSMIC ID
ALK	CLTC	COSF470
ALK	CLTC	COSF434/435
ALK	CLTC	COSF472
ALK	DCTN1	
ALK	EML4	COSF1065/1128
ALK	EML4	COSF1368
ALK	EML4	COSF412
ALK	EML4	COSF476
ALK	EML4	COSF408/463
ALK	EML4	COSF1062/1063
ALK	EML4	COSF1539/1540
ALK	EML4	COSF462
ALK	EML4	COSF410
ALK	EML4	COSF414
ALK	EML4	COSF1541/1542
ALK	EML4	COSF1064
ALK	EML4	COSF1127
ALK	EML4	COSF477/491
ALK	EML4	COSF413
ALK	EML4	COSF475
ALK	EML4	COSF1367
ALK	EML4	
ALK	EML4	COSF487/1376
ALK	EML4	COSF488
ALK	EML4	COSF409/465
ALK	EML4	COSF730/731
ALK	EML4	COSF490
ALK	EML4	COSF464

3' Gene Partner	5' Gene Partner	COSMIC ID
ALK	EML4	COSF478/1543
ALK	EML4	COSF479/480
ALK	EML4	COSF1296/1297
ALK	EML4	COSF411/734
ALK	EML4	COSF1544/1545
ALK	EML4	
ALK	EML4	COSF474
ALK	EML4	COSF493
ALK	KIF5B	COSF1381/1382
ALK	KIF5B	COSF1060
ALK	KIF5B	COSF1061
ALK	KIF5B	COSF1257/1258
ALK	KIF5B	COSF1058/1059
ALK	KLC1	COSF1277
ALK	SQSTM1	
ALK	STRN	COSF1430/1431
ALK	STRN	COSF1669
ALK	STRN	COSF1670
ALK	STRN	COSF1538
ALK	TFG	COSF430
ALK	TFG	COSF432
ALK	TFG	COSF428/429
ALK	TFG	COSF424/425
ALK	TFG	COSF426
ALK	TPM3	COSF439
CIT	FGFR2	
FGFR1	BAG4	
MBIP	AXL	

3' Gene Partner	5' Gene Partner	COSMIC ID
NRG1	CD74	COSF1636
NRG1	CD74	COSF1666
NTRK1	CD74	
NTRK1	MPRIP	
NTRK1	TFG	COSF1328
NTRK1	TPM3	COSF1329/1330
NTRK3	ETV6	COSF571
NTRK3	ETV6	COSF1534
NTRK3	ETV6	COSF823
PDGFRA	SCAF11	
RET	CCDC6	COSF1533
RET	CCDC7	COSF1271
RET	CCDC8	COSF1532
RET	CCDC9	COSF1515
RET	KIF5B	COSF1232
RET	KIF5B	COSF1230
RET	KIF5B	COSF1253
RET	KIF5B	COSF1234
RET	KIF5B	COSF1241
RET	KIF5B	COSF1262
RET	NCOA4	COSF1340
RET	TRIM33	
ROS1	CCDC6	
ROS1	CD74	COSF1478
ROS1	CD74	COSF1202
ROS1	CD74	COSF1200
ROS1	CLTC	
ROS1	EZR	COSF1267

3' Gene Partner	5' Gene Partner	COSMIC ID
ROS1	EZR	COSF1396
ROS1	GOPC	COSF1139
ROS1	GOPC	COSF1140
ROS1	GOPC	COSF1188
ROS1	GOPC	COSF1243
ROS1	LRIG3	COSF1269
ROS1	SDC4	
ROS1	SDC4	COSF1265

3' Gene Partner	5' Gene Partner	COSMIC ID
ROS1	SDC4	COSF1278
ROS1	SDC4	COSF1280
ROS1	SLC34A2	COSF1261
ROS1	SLC34A2	COSF1259
ROS1	SLC34A2	COSF1196
ROS1	SLC34A2	COSF1198
ROS1	TPM3	COSF1273
ROS1	TPM3	

3' Gene Partner	5' Gene Partner	COSMIC ID
TACC3	FGFR3	COSF1434
TACC3	FGFR3	COSF1353
TACC3	FGFR3	COSF1348
TACC3	FGFR3	COSF1350
TACC3	FGFR3	COSF1349
TACC3	FGFR3	COSF1352
TACC3	FGFR3	COSF1357

Note: Shading is intended to assist with visual grouping of the common 3' Gene Partners.

Test Principle

The kit includes reagents for RT, cDNA QC, targeted enrichment, index codes, library purification, quantification, and an easy-to-use bioinformatics software solution. Both wet and dry bench processes are integrated within a simple workflow optimized for use with low-quality and low-quantity RNA/TNA samples isolated from FFPE (Formalin-Fixed, Paraffin-Embedded), FNA (Fine Needle Aspiration) tumor biopsies, fresh frozen (FF) tissue, and cell lines.

Each kit component serves an essential role in the library prep workflow:

- QuantideX® NGS RNA Assay (Lung) – Reagents for functional RNA RT, subsequent quantification, and sample QC assessment. Additionally, the box contains the RNA Fusion Positive Ctrl (Lung).
- QuantideX® NGS RNA Lung Panel – PCR primers and reagents that support single-well multiplex PCR enrichment across all targets.
- QuantideX® NGS Codes: Set A/B/C/D – Dual Index code oligonucleotide mixtures specific for the MiSeq. These dual-index code primer mixes are formulated in racks of 48 codes.
- QuantideX® NGS Library Pure Prep – A proprietary magnetic bead chemistry that provides size selection and purification of the amplified libraries.
- QuantideX® NGS Library Quant – An assay that enables accurate assessment of purified libraries using a quantitative real-time PCR method.
- QuantideX® NGS Sequencing Reagents – Custom sequencing primer mixtures for QuantideX NGS and standard Illumina library analysis.
- QuantideX® NGS Reporter –An easy to use, integrated informatics processing and data reporting pipeline based on proprietary alignment and variant scoring algorithms.

Limitations

Sample Input

- The concentration of the cDNA sample must be determined as functional or amplifiable copies per microliter (cp/µL).
    - Use the QuantideX® NGS RNA Assay to obtain copy number concentrations for each sample's RT product.
    - Samples with < 100 cp/µL may be analyzed, but are at-risk for inaccuracies due to the reduced template diversity available within that sample for amplification.
    - For best results, load ≥ 100 cp/µL (Total: 400 amplifiable copies) but < 6000 cp/µl into the Gene-Specific PCR enrichment.

Rare Single Nucleotide Polymorphisms (SNPs)

- The QuantideX® NGS library preparation method is based on PCR amplification of targeted regions of cancer-associated genes (see Appendix). The presence of rare SNPs in the primer binding region may result in reduced amplicon yield or allele dropout.

Thermal cycler and qPCR Instrument platforms

- The Kit has been verified for use on the ABI 9700 and Veriti instruments with default ramp rates. ***Use of other instrument platforms other than those mentioned here is NOT supported***.[1]
- The RNA Assay and LQ qPCR protocol has been verified for use on the ABI 7500, ABI 7500 Fast in standard mode and Roche LC480 & Cobas Z480 instruments. ***Analysis in fast mode or use of other instrument platforms other than those mentioned here is NOT supported***.[2]

Reagent Stability

- The reagents should be used within their labeled expiry and stored according to kit component table.
- Reagents stored frozen (-15 to -30°C) are formulated to support eight (8) freeze-thaw cycles.

MiSeq Instrument Control

- The PhiX sequencing control is highly recommended for inclusion in each NGS run for cluster diversification and to aid in troubleshooting.

Instrument/Platform Compatibility

- This protocol was written to support MiSeq instrument analysis. Specific modifications may be required for other Illumina Sequencing platforms (*e.g.* NextSeq system).
- A separate protocol guide may be used to describe use of these reagents for alternative platforms.

Warnings and Precautions

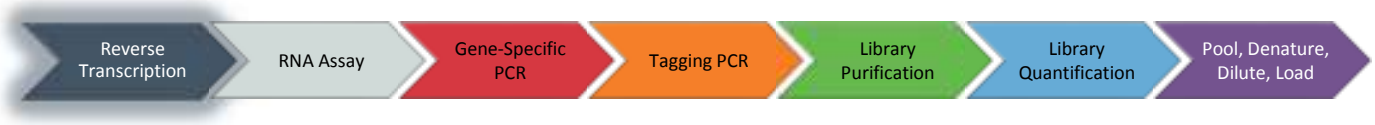
- Use proper PPE. Wear appropriate protective eyeglasses, protective gloves, and protective clothing when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910:1030, CLSI M29, or other applicable guidance when handling human samples.
- Use nuclease-free filter pipette tips and nuclease-free tubes.
- Seal plates in a safe and appropriate manner to reduce likelihood of evaporation or cross contamination of wells during library preparation. Automated heat sealing using peelable foil seals is recommended.
    - The BioRad PX1 Heat Sealer (185°C, 3 seconds) with Eppendorf Twin-Tec 96 well plates and peelable foil heat seals (BioRad Ref #1814045), or equivalent, has been verified for efficacy.
- PCR carry-over contamination can result in false-positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
    - ⚠ Separation of template *(i.e.* sample handling) and non-template *(i.e.* master mix formulation) handling is highly recommended to reduce the probability of introducing contamination into the workflow.
- Do not combine components from different reagent lots.
- Do not let beads dry out during library Purification to mitigate risk of sample loss/low library yield.
    - ⚠ To reduce drying effects, divide the plate into sets of 2 or 3 columns at a time when purifying for large sample batches.
- Prior to use, ensure that all instruments are calibrated according to the manufacturer's instructions.
- **When working with less than 8 samples, it is recommended to repeat Library Quant after pooling/normalizing libraries prior to loading the MiSeq.**

[1] *If interested in using a different library quantitation (qPCR) platform, please contact Asuragen Tech Support for guidance.*

QuantideX® NGS RNA Lung Cancer Kit Library Prep Protocol

Overview/Notes

- Track sample ID, amplifiable copy number, and assigned index code carefully throughout the procedure. ⚠ All three are critical for successful sequencing analysis.
- Use this protocol in conjunction with Illumina's MiSeq System User Guide.
- All master mixes and reactions may be prepared at room temperature.
- When combining reagents as instructed, pipette up and down 3-5 times with each addition to ensure a full dispense.
- Replicate testing of samples in qPCR assays is not required or recommended.
- Library prep can be safely stopped after each major reaction in the procedure.
    - After the reaction is complete, store sample plate(s) at 2 to 8°C for short term (≤ 24 hrs) or long term at -15 to -30°C. Keep all reaction plates until sequencing data has been obtained.
    - When ready to proceed, bring plate to room temperature with other required reagents.
- Prior to each reaction,
    - Bring each required reagent and/or plate to room temperature for at least 15 minutes.
    - Briefly centrifuge each plate before use.
    - Briefly vortex and centrifuge each component prior to opening.
      ⚠ Exception: Do not centrifuge the ● Library Pure Prep Beads.



Reverse Transcription (RT) [Box 1]

Instrument run time: 55 min

1. Prepare an RT reaction master mix in a clean microcentrifuge tube.
    A. Add the reagents to the tube in the order listed; volumes are shown per reaction.
    B. Prepare sufficient master mix for the total number of samples tested, including any necessary controls (NTC and ● RNA Fusion Positive Ctrl).
    C. Mix the master mix with gentle flicking/vortexing, briefly centrifuge to collect contents.

Description	Vol. (µL)
● QuantideX® RT Buffer	4
● QuantideX® RT Enzyme Mix	1
Total master mix per well	5

2. Aliquot 5 µL of RT master mix to separate wells in a 96-well reaction plate.
3. Add 5 µL of each RNA/TNA sample of interest to separate wells containing RT master mix, mix by pipetting.
4. Add 5 µL of each control to separate wells containing RT master mix, mix by pipetting.
    A. ● RNA Fusion Positive Ctrl
    B. NTC (nuclease-free water)
5. Seal the plate and briefly centrifuge to collect contents.
   ⚠ Do not vortex plate to reduce risk of contamination.
6. Perform the 10 µL Reverse Transcription (RT) reaction on a thermal cycler with the following conditions:

Temp.	Time
42°C	45 min
93°C	10 min
4°C	hold

Including the suggested controls in each library prep batch helps to ensure run validity, monitor operator proficiency, and track performance trends over time.

RNA Assay [Box 1]

Instrument run time: 1.5 hours

1. Prepare a qPCR master mix in a clean microcentrifuge tube.
   A. Add the reagents to the tube in the order listed; volumes are shown per reaction.
   B. Prepare sufficient master mix for the total number of RT products, plus the 4 qPCR standards in duplicate (# of RT products + 8).
   C. Mix the master mix with gentle flicking/vortexing, then pulse spin to collect contents.
   D. After first use, store ● 20X ROX at 2 to 8°C

Description	Vol. (µL)
● 2X qPCR Master Mix	5.0
● Primer/Probe Mix	2.5
● 20X ROX†	0.5
Total master mix per well	8.0

† If using a qPCR instrument that does not utilize a passive reference, substitute this volume with nuclease-free water.

2. Aliquot 8 µL of qPCR master mix to separate wells in a 96-well optical PCR plate.
3. Add 2 µL of each ○ qPCR Standard to separate wells in duplicate (8 wells total) and mix by pipetting.
4. Add 2 µL of each RT product to separate wells and mix by pipetting.
5. Seal plate with optical seal, then centrifuge plate to collect contents.
   ⚠ Do not vortex plate to reduce risk of contamination.
6. Perform the 10 µL qPCR reaction on a qPCR instrument with parameters below:
   - Collect data during 60°C step
   - ABI 7500 instruments
     - Standard mode
     - FAM detector, no quencher,
     - ROX Passive Reference;
     - Auto Baseline;
     - Manual Threshold: 0.3
   - Roche LC480 instruments:
     - FAM detector
     - Fit Points analysis
     - Suggested analysis setting (verify and adjust per run):
       Background 3-9,
       Noise band = STD Multiplier 36,
       Threshold = Auto;

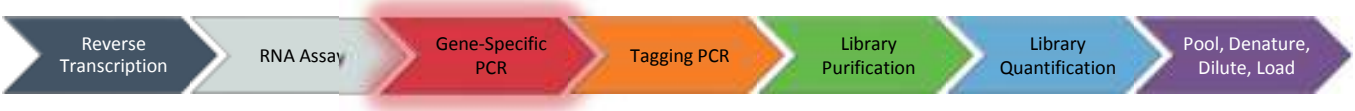
Temp.	Time	Cycling
95°C	5 min	-
95°C	5 sec	40 cycles
60°C	60 sec	

Example of a 6-reaction plate layout:

	1	2	3
A	qPCR Std. 1	Sample1	
B	qPCR Std. 1	Sample2	
C	qPCR Std. 2	Sample3	
D	qPCR Std. 2	Sample4	
E	qPCR Std. 3	RNA Fusion Pos. Ctrl.	
F	qPCR Std. 3	NTC	
G	qPCR Std 4		
H	qPCR Std 4		

7. Analysis
   A. Determine Copy Input
   Plot a standard curve of the qPCR Standards, using log (concentration) as the x-axis, and FAM Cq values for each standard as the y-axis.
   Using the equation of the best-fit linear line, calculate concentration (cp/µL) for each sample.
   ⚠ Record all sample copy numbers for analysis by the QuantideX® Reporter.
   B. Input recommendations into QuantideX® NGS RNA Lung library prep:
   < 100 cp/µL: may proceed to library prep, but at-risk for call inaccuracies due to potentially limiting functional template quantities
   ≥ 100 cp/µL: recommended input for library prep
   > 6000 cp/µL: dilute an aliquot of the sample 10-fold in nuclease-free water for use in library Prep (e.g. 2 µL sample in 18 µL water).

Description	Conc. (cp/µL)
qPCR Standard 1 (Lung)	6250
qPCR Standard 2 (Lung)	1250
qPCR Standard 3 (Lung)	250
qPCR Standard 4 (Lung)	50



Gene-Specific PCR [Box 2]

Instrument run time: 2.5 hours

1. Prepare a GS PCR master mix in a clean microcentrifuge tube.
   A. Add the reagents to the tube in the order listed; volumes are shown per reaction.
   B. Create enough master mix for all RT products (samples of interest plus any controls included).
   C. Gently vortex to mix, and briefly centrifuge the GS PCR master mix.

Description	Vol. (µL)
2X Amplification Master Mix	5
RNA Lung Primer Mix	1
Total master mix per well	6

2. Aliquot 6 µL of the GS PCR master mix into separate wells of a clean 96-well plate.
3. Add 4 µL of each RT product to separate wells containing GS PCR master mix, mix by pipetting.
   Note: Recommended cDNA input range is > 400 copies (>100 cp/µL)
4. Seal the plate, briefly centrifuge the plate to collect contents.
   ⚠ Automated heat sealing using peelable foil seals is recommended.
   ⚠ Do not vortex plate to reduce risk of contamination.
5. Perform the 10 µL GS PCR reaction on a thermal cycler with the indicated cycling conditions.

Temp.	Time	Cycling
95°C	5 min	-
95°C	15 sec	2 cycles
60°C	4 min	
95°C	15 sec	23 cycles
72°C	4 min	
72°C	10 min	-
4°C	hold	-

Tagging PCR [Box 2 and Box 3]

Instrument run time: 45 min

1. Preparation
   A. Centrifuge the rack of index codes for 1 minute at 2000 x g prior to each use.
   B. If needed, scan rack of codes with a barcode scanner to ensure correct positions of index codes. Refer to the provided COA as needed.
   C. Assign each sample/control from GS PCR a unique Index Code (AIL001-AIL192), and record this information by plate location and sample ID.
   ⚠ *Critical: Each sample must be assigned (and receive) a different index code.*
2. In a new 96-well plate, for each GS PCR product:
   A. Aliquot 7.5 µL 2X Index Master Mix
   B. Add 5.5 µL Index Code (AIL###, unique to each well)
3. Following brief centrifugation, transfer 2 µL GS PCR product to corresponding wells containing Index Codes and Master Mix. Mix by pipetting.

⚠ *Important: Ensure the correct Index Code was added to the appropriate well according to the previously-made assignments and that each well received a different Index Code.*

4. Seal the plate, and briefly centrifuge to collect contents.
   - ⚠ Automated heat sealing using peelable foil seals is recommended.
   - ⚠ Do not vortex plate to reduce risk of contamination.

5. Perform the 15 µL Tagging PCR reaction on a thermal cycler with the indicated cycling conditions.

Temp.	Time	Cycling
95°C	5 min	-
95°C	30 sec	10 cycles
55°C	30 sec	
72°C	60 sec	
72°C	10 min	-
4°C	hold	-

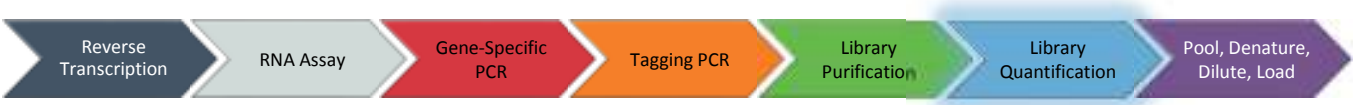
Library Purification [Box 4]

1. Initial Setup
   - A. If this is the first use of the kit, add 10 mL of 100% Ethanol to Wash Buffer bottle, cap the bottle, and mix well by inverting the bottle several times.
     - ⚠ 10 mL of 100% ethanol must be added to the Wash Buffer bottle.
     - ⚠ Failure to add ethanol to the Wash Buffer will result in sample and reagent loss.
   - B. Bring ● Library Pure Prep Beads to room temperature prior to use
   - C. Fully re-suspend ● Library Pure Prep Beads with *gentle* vortexing for 30 - –5 seconds.
     - ⚠ *DO NOT centrifuge the ● Library Pure Prep Beads.*
   - D. Ensure reagents and consumables are readily available before starting the purification. It is important to not let the beads dry out until the very end of the procedure.
     - ⚠ To reduce drying effects, divide the plate into sets of 2 or 3 columns at a time when doing the purification for large sample batches.
2. Bind the Tag PCR product to the magnetic beads
   - A. Aliquot 11 µL ● Library Pure Beads to each well in a clear, round-bottom, 96-well plate (Evergreen Scientific: 290-8117-01R). *Prime pipette tip to ensure full volume dispense.*
     Note: To keep bead concentration consistent across the plate while aliquotting, intermittently cap the ● Library Pure Prep Beads after every 4 wells and gently pulse vortex.
   - B. After brief centrifugation, add 10 µL Tag PCR products to the wells of beads, mix by pipetting until mixture appears homogenous. 5 µL Tag PCR product will remain in Tag PCR plate.
     Note: Avoid creating bubbles while mixing.
     Note: Recommended use of multichannel for ease of work flow from this point onwards.

3. Wash the bead-bound libraries:
   - A. Place plate on magnetic stand (Ambion AM10027) to pellet beads out of solution (≈15 seconds).
   - B. Leaving the plate on the magnetic stand, slowly remove and discard the clear supernatant (≈20 µL) from each well, taking care to avoid the bead pellet.
   - C. Leaving the plate on the magnetic stand, add 100 µL of Wash Buffer containing Ethanol to each well.
   - D. Wait for beads to re-pellet if they were disrupted when adding the Wash Buffer (≈15 seconds).
   - E. Leaving the plate on the magnetic stand, slowly remove and discard the clear supernatant from each well, taking care to avoid the bead pellet.

F. Repeat steps C to E to perform another wash for a total of two washes, removing as much buffer as possible after the second wash.

4. Elute the purified libraries:
   A. After removal of all Wash Buffer, incubate plate on the magnet at room temperature for 1 minute to dry the beads.
   Note: Excessive drying of beads (> 2 min) may lead to loss of product.
   B. Remove the plate from the magnet.
   C. Add 20 µL of ● Elution Buffer to each well and mix by pipetting until bead pellets are fully re-suspended and elution reaction appears homogenous.
   ⚠ *Use pipette tip in excess of 20 µL to avoid aspirating mixture into tip filters.*
   Notes: Beads may clump or appear granular in solution during this step. This is acceptable, and does not affect the elution.
   D. Incubate plate for 5 minutes on bench top once all bead pellets are re-suspended.
   E. Place plate on magnet to pellet beads out of solution (≈15 seconds).
   F. Transfer 18 µL of the clear supernatant into a clean 96-well plate, taking care to avoid the beads. This resultant plate contains the purified libraries to be pooled. An aliquot of the purified libraries will be quantified (see next step) prior to pooling.



Library Quantification [Box 5]

Instrument run time: 1.5 hours

1. Dilute an aliquot of purified library 10,000-fold (via two 100-fold serial dilutions) in LQ Diluent using the following procedure. *(Note: Both dilutions are discarded after the products have been quantified.)*
   ⚠ Thaw LQ Diluent at least 30 min prior to use.
   A. Transfer 198 µL LQ Diluent to each well in a 96-well plate.
   B. Add 2 µL of purified library, pipette up and down 5 times to ensure a full dispense.
   C. Mix by pipetting 10-20 times with a pipet set to 150 µL.
   D. Add 198 µL LQ Diluent to clean wells in a second 96-well plate.
   E. Transfer 2 µL of 1:100 diluted library to second plate, pipette up and down 5 times to ensure a full dispense.
   F. Mix by pipetting 10-20 times with a pipet set to 150 µL.
   G. After first use, store LQ Diluent at 2 to 8°C.
2. Prepare an LQ qPCR master mix in a clean microcentrifuge tube.
   A. Add the reagents to the tube in the order listed; volumes are shown per reaction.

Description	Vol. (µL)
● 2X qPCR Master Mix	5
● LQ Primer/Probe Mix	2.5
● 20X ROX†	0.5
Total master mix per well	8.0

† If using a qPCR instrument that does not utilize a passive reference, substitute this volume with nuclease-free water.

   B. Create enough master mix for all 10,000-fold diluted purified products, plus the 4 LQ standards in duplicate (# of diluted products + 8).
   C. Gently vortex the tube to mix, and briefly centrifuge the LQ qPCR master mix.
   D. After first use, store ● 20X ROX at 2 to 8°C.

Example of a 6-sample plate layout:

	1	2	3
A	LQ Std. 1	Sample1	
B	LQ Std. 1	Sample2	
C	LQ Std. 2	Sample3	
D	LQ Std. 2	Sample4	
E	LQ Std. 3	Sample5	
F	LQ Std. 3	Sample6	
G	LQ Std. 4		
H	LQ Std. 4		

3. Combine LQ qPCR master mix with standards and diluted samples in a 96-well optical plate.
    A. Aliquot 8 µL LQ qPCR master mix into a 96-well optical plate.
    B. Add 2 µL of each LQ Standard to separate wells in duplicate (8 wells total) and mix by pipetting.
    C. Add 2 µL of 10,000-fold diluted purified products to separate wells and mix by pipetting.
    D. Seal the plate with an optical seal compatible with the qPCR instrument to be used and briefly centrifuge to collect contents.
4. Perform 10 µL LQ PCR on a qPCR instrument with the following conditions/parameters:
    - Collect data during 60°C step
    - ABI 7500 instruments
        - Standard mode
        - FAM detector, no quencher
        - ROX Passive Reference
        - Auto Baseline
        - Manual Threshold: 0.3

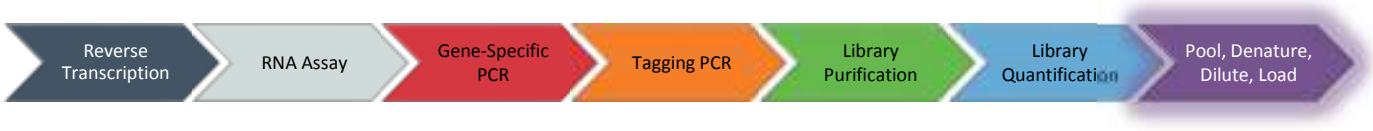
Temp.	Time	Cycling
95°C	5 min	-
95°C	5 sec	35 cycles
60°C	60 sec	

    - Roche LC480 instruments:
        - FAM detector
        - Fit Points analysis
        - Suggested analysis setting (verify and adjust per run):
            Background 3-9
            Noise band = STD Multiplier 36
            Threshold = Auto

5. Analysis
    A. Plot a standard curve of the LQ Standards using log (concentration) as the x-axis and FAM Cq values of each standard as the y-axis.
    B. Using the equation of the best-fit linear line, calculate concentration (pM) for the diluted purified products.
    C. Multiply concentrations by the dilution factor (10,000) to derive the concentrations of the stock purified products.
    D. Divide the concentrations by 1,000 to convert from pM to nM.
    E. Use these concentrations in nM of the purified products for future steps.

Description	*Conc. (pM)*
LQ Standard 1	20
LQ Standard 2	2
LQ Standard 3	0.2
LQ Standard 4	0.02

⚠ **<u>Discard all dilution plates before proceeding to the next step.</u>**



Pool, Denature, Dilute, Load [Box 6]

Use the following instructions in conjunction with Illumina's instructions for loading and running the MiSeq.

1. Setup
    A. Follow the manufacturer's (Illumina) instructions for thawing and preparing the MiSeq Reagent cartridge for loading.
    B. If needed, dilute stock PhiX control to 1 nM in ● Sequencing Diluent.
    C. If needed, dilute stock NaOH to 1 N in nuclease-free water.
2. Pooling/normalizing the libraries based on their respective concentrations from LQ PCR:
    A. Determine the median concentration of all the prepared libraries, $Conc_{median}$.
    B. Use the formula below to determine how much volume of each individual sample library to add to the pool. The formula targets 5 µL per library.

$$Vol_{lib} = \frac{Conc_{median}}{Conc_{lib}} * 5\ \mu L$$

⚠ *For ease of pipetting, round the calculated volume to the nearest microliter.*

    C. Transfer the calculated, rounded volume of each library to a clean microcentrifuge tube.
    D. Do not pipet less than 2 µL of any one library and no more than 15 µL.
    E. For NTCs, transfer only 4 µL of purified product, regardless of the calculated concentration.
3. Using the rounded volumes used of each library, calculate the total concentration of the pool:

$$Conc_{pool} = \frac{(Conc_{lib} * Vol_{lib})_1 + (Conc_{lib} * Vol_{lib})_i}{Vol_{pool}}$$ *where "i" is each library*

⚠ *When working with less than 8 samples, it is recommended to repeat Library Quant after pooling/normalizing libraries prior to loading the MiSeq.*

4. Dilute the library pool to 2.5 nM in ● Sequencing Diluent in a clean microcentrifuge tube.
5. Transfer 992 µL of the HT1-hyb buffer to a clean microcentrifuge tube, place on ice.
6. Denature the pooled library:
    A. Transfer 15 µL of the 2.5 nM pool to a clean microcentrifuge tube at room temperature.
    B. Add 3 µL of 1 nM PhiX control, vortex to mix, briefly centrifuge.
    C. Add 2 µL of 1 N NaOH, immediately cap the tube, vortex to mix, briefly centrifuge.
    D. Incubate the tube for 5 minutes at room temperature.
    E. Place tube on ice immediately for at least 2 minutes. This tube now holds the denatured library.
7. Dilute the denatured library mixture:
    A. Transfer 8 µL of the denatured library to the pre-chilled 992 µL aliquot of HT1-hyb buffer.
    B. Vortex to mix, briefly centrifuge to collect contents, place back on ice. (This 1000 µL is the sample to be loaded into the MiSeq cartridge in position 17.)
8. Dilute the sequencing primers for loading onto the MiSeq in 3 separate microcentrifuge tubes on ice:
    A. Add 4 µL ● Read 1 Sequencing Primers to 636 µL HT1-hyb buffer.
    B. Add 4 µL ● Index Read Sequencing Primers to 636 µL HT1-hyb buffer.
    C. Add 4 µL ● Read 2 Sequencing Primers to 636 µL HT1-hyb buffer.
    D. Vortex all to mix, briefly centrifuge, place on ice until ready to load into MiSeq cartridge.
9. Load the MiSeq cartridge:
    A. Add 600 µL of the pooled, denatured, diluted library to position 17.
    B. Add 600 µL diluted Read 1 Sequencing Primers to position 18.
    C. Add 600 µL diluted Index Read Sequencing Primers to position 19.
    D. Add 600 µL diluted Read 2 Sequencing Primers to position 20.
    ⚠ Follow manufacturer's (Illumina) instructions on loading and running the MiSeq instrument.

Generation of MiSeq Sample Sheet

1. Download Illumina's IEM software at Illumina.com.
2. Add the "ASGN QuantideX NGS RNA Lung.txt" protocol file into the appropriate location to integrate with the IEM software (e.g.: C:\Program Files\Illumina\Illumina Experiment Manager\SamplePrepKits).
3. Replace the "Generate FASTQ.txt" file found in <C:\Program Files\Illumina\Illumina Experiment Manager\Applications> with the Asuragen-provided version of the .txt file.
   Note: Both of the .txt files mentioned above are included with the purchase of QuantideX® NGS RNA Lung Cancer products, and also available at quantidex.asuragen.com.
4. Follow Illumina's instructions to generate the sample sheet in the IEM software, adhering to the following parameters:
   - Sample Prep Kit Type: ASGN QuantideX NGS RNA Lung
   - Index Reads: 2
   - Assign Index Codes (AIL001-192) I-5 and I-7 to correct sample IDs, making sure they match the assignments made during Tagging/Index PCR. Refer to QuantideX® NGS Codes COA as needed.
   - Instrument: MiSeq
   - Application Category: Other
   - Application: FASTQ only
   - FASTQ only run settings:
     - Cycles Read 1: 201
     - Cycles Read 2: 201
   - FASTQ only Workflow-Specific settings:
     - Custom Primer for Read 1
     - Custom Primer for Index
     - Custom Primer for Read 2
5. Transfer the resulting .csv file to the MiSeq instrument, follow Illumina's guide on running the MiSeq.

Data Analysis

A completely integrated bioinformatics suite, the QuantideX® NGS Reporter software (49562), is available with purchase of the kit at http://quantidex.asuragen.com along with QuantideX® NGS Reporter User Guide (PC-0249). The patent-pending software requires the copy input for each sample determined from the RNA Assay to inform the variant caller.

Expected Results of Kit Controls/Standards and QC Steps

- RNA Assay and Library Quant Standards: coefficient of determination (r^2) > 0.96
- RNA Fusion Positive Ctrl (Lung): EML4_ALK fusion detected (annotated as Synthetic)

Notice to Purchaser

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- The QuantideX® Library Quant Kit was optimized with ABI 7500, 7500 FastDX, Roche LC480, and Cobas Z480 real-time instruments. Contact Asuragen Tech Support for alternative quantification methods.
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Symbol Legend

Symbol	Description
	Catalog number
	Batch code
	Contains sufficient reagents for N reactions
	Consult instructions before use
	Temperature limitation
	Use by
	Manufactured by

Reference

Hadd, A.G., et al., J Mol Diagn, 2013. 15(2): p. 234-47.

Appendix 1: Index Codes

QuantideX® NGS Codes - Set A 49553 ; 150004			**QuantideX® NGS Codes - Set B 49554 ; 150005**			**QuantideX® NGS Codes - Set C 49555 ; 150006**			**QuantideX® NGS Codes - Set D 49556 ; 150007**		
Set ID	**I7 Index ID**	**I5 Index ID**	**Set ID**	**I7 Index ID**	**I5 Index ID**	**Set ID**	**I7 Index ID**	**I5 Index ID**	**Set ID**	**I7 Index ID**	**I5 Index ID**
AIL001	I7-001	I5-01	AIL049	I7-007	I5-01	AIL097	I7-013	I5-01	AIL145	I7-019	I5-01
AIL002	I7-001	I5-02	AIL050	I7-007	I5-02	AIL098	I7-013	I5-02	AIL146	I7-019	I5-02
AIL003	I7-001	I5-03	AIL051	I7-007	I5-03	AIL099	I7-013	I5-03	AIL147	I7-019	I5-03
AIL004	I7-001	I5-04	AIL052	I7-007	I5-04	AIL100	I7-013	I5-04	AIL148	I7-019	I5-04
AIL005	I7-001	I5-05	AIL053	I7-007	I5-05	AIL101	I7-013	I5-05	AIL149	I7-019	I5-05
AIL006	I7-001	I5-06	AIL054	I7-007	I5-06	AIL102	I7-013	I5-06	AIL150	I7-019	I5-06
AIL007	I7-001	I5-07	AIL055	I7-007	I5-07	AIL103	I7-013	I5-07	AIL151	I7-019	I5-07
AIL008	I7-001	I5-08	AIL056	I7-007	I5-08	AIL104	I7-013	I5-08	AIL152	I7-019	I5-08
AIL009	I7-002	I5-01	AIL057	I7-008	I5-01	AIL105	I7-014	I5-01	AIL153	I7-020	I5-01
AIL010	I7-002	I5-02	AIL058	I7-008	I5-02	AIL106	I7-014	I5-02	AIL154	I7-020	I5-02
AIL011	I7-002	I5-03	AIL059	I7-008	I5-03	AIL107	I7-014	I5-03	AIL155	I7-020	I5-03
AIL012	I7-002	I5-04	AIL060	I7-008	I5-04	AIL108	I7-014	I5-04	AIL156	I7-020	I5-04
AIL013	I7-002	I5-05	AIL061	I7-008	I5-05	AIL109	I7-014	I5-05	AIL157	I7-020	I5-05
AIL014	I7-002	I5-06	AIL062	I7-008	I5-06	AIL110	I7-014	I5-06	AIL158	I7-020	I5-06
AIL015	I7-002	I5-07	AIL063	I7-008	I5-07	AIL111	I7-014	I5-07	AIL159	I7-020	I5-07
AIL016	I7-002	I5-08	AIL064	I7-008	I5-08	AIL112	I7-014	I5-08	AIL160	I7-020	I5-08
AIL017	I7-003	I5-01	AIL065	I7-009	I5-01	AIL113	I7-015	I5-01	AIL161	I7-021	I5-01
AIL018	I7-003	I5-02	AIL066	I7-009	I5-02	AIL114	I7-015	I5-02	AIL162	I7-021	I5-02
AIL019	I7-003	I5-03	AIL067	I7-009	I5-03	AIL115	I7-015	I5-03	AIL163	I7-021	I5-03
AIL020	I7-003	I5-04	AIL068	I7-009	I5-04	AIL116	I7-015	I5-04	AIL164	I7-021	I5-04
AIL021	I7-003	I5-05	AIL069	I7-009	I5-05	AIL117	I7-015	I5-05	AIL165	I7-021	I5-05
AIL022	I7-003	I5-06	AIL070	I7-009	I5-06	AIL118	I7-015	I5-06	AIL166	I7-021	I5-06
AIL023	I7-003	I5-07	AIL071	I7-009	I5-07	AIL119	I7-015	I5-07	AIL167	I7-021	I5-07
AIL024	I7-003	I5-08	AIL072	I7-009	I5-08	AIL120	I7-015	I5-08	AIL168	I7-021	I5-08
AIL025	I7-004	I5-01	AIL073	I7-010	I5-01	AIL121	I7-016	I5-01	AIL169	I7-022	I5-01
AIL026	I7-004	I5-02	AIL074	I7-010	I5-02	AIL122	I7-016	I5-02	AIL170	I7-022	I5-02
AIL027	I7-004	I5-03	AIL075	I7-010	I5-03	AIL123	I7-016	I5-03	AIL171	I7-022	I5-03
AIL028	I7-004	I5-04	AIL076	I7-010	I5-04	AIL124	I7-016	I5-04	AIL172	I7-022	I5-04
AIL029	I7-004	I5-05	AIL077	I7-010	I5-05	AIL125	I7-016	I5-05	AIL173	I7-022	I5-05
AIL030	I7-004	I5-06	AIL078	I7-010	I5-06	AIL126	I7-016	I5-06	AIL174	I7-022	I5-06
AIL031	I7-004	I5-07	AIL079	I7-010	I5-07	AIL127	I7-016	I5-07	AIL175	I7-022	I5-07
AIL032	I7-004	I5-08	AIL080	I7-010	I5-08	AIL128	I7-016	I5-08	AIL176	I7-022	I5-08
AIL033	I7-005	I5-01	AIL081	I7-011	I5-01	AIL129	I7-017	I5-01	AIL177	I7-023	I5-01
AIL034	I7-005	I5-02	AIL082	I7-011	I5-02	AIL130	I7-017	I5-02	AIL178	I7-023	I5-02
AIL035	I7-005	I5-03	AIL083	I7-011	I5-03	AIL131	I7-017	I5-03	AIL179	I7-023	I5-03
AIL036	I7-005	I5-04	AIL084	I7-011	I5-04	AIL132	I7-017	I5-04	AIL180	I7-023	I5-04
AIL037	I7-005	I5-05	AIL085	I7-011	I5-05	AIL133	I7-017	I5-05	AIL181	I7-023	I5-05
AIL038	I7-005	I5-06	AIL086	I7-011	I5-06	AIL134	I7-017	I5-06	AIL182	I7-023	I5-06
AIL039	I7-005	I5-07	AIL087	I7-011	I5-07	AIL135	I7-017	I5-07	AIL183	I7-023	I5-07
AIL040	I7-005	I5-08	AIL088	I7-011	I5-08	AIL136	I7-017	I5-08	AIL184	I7-023	I5-08
AIL041	I7-006	I5-01	AIL089	I7-012	I5-01	AIL137	I7-018	I5-01	AIL185	I7-024	I5-01
AIL042	I7-006	I5-02	AIL090	I7-012	I5-02	AIL138	I7-018	I5-02	AIL186	I7-024	I5-02
AIL043	I7-006	I5-03	AIL091	I7-012	I5-03	AIL139	I7-018	I5-03	AIL187	I7-024	I5-03
AIL044	I7-006	I5-04	AIL092	I7-012	I5-04	AIL140	I7-018	I5-04	AIL188	I7-024	I5-04
AIL045	I7-006	I5-05	AIL093	I7-012	I5-05	AIL141	I7-018	I5-05	AIL189	I7-024	I5-05
AIL046	I7-006	I5-06	AIL094	I7-012	I5-06	AIL142	I7-018	I5-06	AIL190	I7-024	I5-06
AIL047	I7-006	I5-07	AIL095	I7-012	I5-07	AIL143	I7-018	I5-07	AIL191	I7-024	I5-07
AIL048	I7-006	I5-08	AIL096	I7-012	I5-08	AIL144	I7-018	I5-08	AIL192	I7-024	I5-08

Index region sequences of the Index Codes can be found in the ASGN QuantideX NGS RNA Lung.txt file distributed with the purchase of the kit, or available at http://quantidex.asuragen.com.

QuantideX® NGS Codes Rack Layouts

QuantideX® NGS Codes - Set A (49553; 150004)

		I7 Index ID											
		I7-001	*I7-002*	*I7-003*	*I7-004*	*I7-005*	*I7-006*						
I5 Index ID		1	2	3	4	5	6	7	8	9	10	11	12
I5-01	A	AIL001	AIL009	AIL017	AIL025	AIL033	AIL041						
I5-02	B	AIL002	AIL010	AIL018	AIL026	AIL034	AIL042						
I5-03	C	AIL003	AIL011	AIL019	AIL027	AIL035	AIL043						
I5-04	D	AIL004	AIL012	AIL020	AIL028	AIL036	AIL044						
I5-05	E	AIL005	AIL013	AIL021	AIL029	AIL037	AIL045						
I5-06	F	AIL006	AIL014	AIL022	AIL030	AIL038	AIL046						
I5-07	G	AIL007	AIL015	AIL023	AIL031	AIL039	AIL047						
I5-08	H	AIL008	AIL016	AIL024	AIL032	AIL040	AIL048						

QuantideX® NGS Codes - Set B (49554; 150005)

		I7 Index ID											
		I7-007	*I7-008*	*I7-009*	*I7-010*	*I7-011*	*I7-012*						
I5 Index ID		1	2	3	4	5	6	7	8	9	10	11	12
I5-01	A	AIL049	AIL057	AIL065	AIL073	AIL081	AIL089						
I5-02	B	AIL050	AIL058	AIL066	AIL074	AIL082	AIL090						
I5-03	C	AIL051	AIL059	AIL067	AIL075	AIL083	AIL091						
I5-04	D	AIL052	AIL060	AIL068	AIL076	AIL084	AIL092						
I5-05	E	AIL053	AIL061	AIL069	AIL077	AIL085	AIL093						
I5-06	F	AIL054	AIL062	AIL070	AIL078	AIL086	AIL094						
I5-07	G	AIL055	AIL063	AIL071	AIL079	AIL087	AIL095						
I5-08	H	AIL056	AIL064	AIL072	AIL080	AIL088	AIL096						

QuantideX® NGS Codes - Set C (49555; 150006)

		I7 Index ID											
		I7-013	*I7-014*	*I7-015*	*I7-016*	*I7-017*	*I7-018*						
I5 Index ID		1	2	3	4	5	6	7	8	9	10	11	12
I5-01	A	AIL097	AIL105	AIL113	AIL121	AIL129	AIL137						
I5-02	B	AIL098	AIL106	AIL114	AIL122	AIL130	AIL138						
I5-03	C	AIL099	AIL107	AIL115	AIL123	AIL131	AIL139						
I5-04	D	AIL100	AIL108	AIL116	AIL124	AIL132	AIL140						
I5-05	E	AIL101	AIL109	AIL117	AIL125	AIL133	AIL141						
I5-06	F	AIL102	AIL110	AIL118	AIL126	AIL134	AIL142						
I5-07	G	AIL103	AIL111	AIL119	AIL127	AIL135	AIL143						
I5-08	H	AIL104	AIL112	AIL120	AIL128	AIL136	AIL144						

QuantideX® NGS Codes - Set D (49556; 150007)

		I7 Index ID											
		I7-019	*I7-020*	*I7-021*	*I7-022*	*I7-023*	*I7-024*						
I5 Index ID		1	2	3	4	5	6	7	8	9	10	11	12
I5-01	A	AIL145	AIL153	AIL161	AIL169	AIL177	AIL185						
I5-02	B	AIL146	AIL154	AIL162	AIL170	AIL178	AIL186						
I5-03	C	AIL147	AIL155	AIL163	AIL171	AIL179	AIL187						
I5-04	D	AIL148	AIL156	AIL164	AIL172	AIL180	AIL188						
I5-05	E	AIL149	AIL157	AIL165	AIL173	AIL181	AIL189						
I5-06	F	AIL150	AIL158	AIL166	AIL174	AIL182	AIL190						
I5-07	G	AIL151	AIL159	AIL167	AIL175	AIL183	AIL191						
I5-08	H	AIL152	AIL160	AIL168	AIL176	AIL184	AIL192						

Note: The codes are based on separate dual index code combinations and are interchangeable for analysis.

Appendix 2: Targeted Regions

3p Gene	5p Gene	Transcript Definition	Type
ALK	CLTC	CLTC{ENST00000269122}:r.1_5101_insGGUG_ALK{ENST00000389048}:r.4080-120_6220	cDNA
ALK	CLTC	CLTC{ENST00000269122}:r.1_5177_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	CLTC	CLTC{ENST00000269122}:r.1_5177+2010_ALK{ENST00000389048}:r.4080-48_6220	cDNA
ALK	DCTN1	DCTN1{ENST00000361874}:r.1_3514_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1903_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2229+2522_ALK{ENST00000389048}:r.4126_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929_EML4{ENST00000318522}:r.903+188_903+220_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929+(7320)_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751_ALK{ENST00000389048}:r.4080-69_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751_ALK{ENST00000389048}:r.4080-90_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751+(3600)_ALK{ENST00000389048}:r.4080-297_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751+1485_ALK{ENST00000389048}:r.4080-1254_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1751+2575_ALK{ENST00000389048}:r.4080-203_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1903_ALK{ENST00000389048}:r.4080-124_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1903_ALK{ENST00000389048}:r.4094_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1903_ALK{ENST00000389048}:r.4118_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1903_insAUAUGCUGGAU_ALK{ENST00000389048}:r.4129_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_1969_ALK{ENST00000389048}:r.4151_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2029_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2229_EML4{ENST00000318522}:r.2229+2517_2229+2522_ALK{ENST00000389048}:r.4126_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2229_insCAUACUAUGUAUACAAGGGAGUU_ALK{ENST00000389048}:r.4126_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2318_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2318+654_insU_ALK{ENST00000389048}:r.4080-172_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2504_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2504_ALK{ENST00000389048}:r.4080-18_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2504+182_ALK{ENST00000389048}:r.4080-67_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_2504+545_ALK{ENST00000389048}:r.4080-232_6220	gDNA
ALK	EML4	EML4{ENST00000318522}:r.1_470_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_470_ALK{ENST00000389048}:r.4080-117_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929_ALK{ENST00000389048}:r.3975_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929_ALK{ENST00000389048}:r.4080-18_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929_insCAAAAAUGUCAACUCGCAAAAAAAACAGCCAAG_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929+220_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	EML4	EML4{ENST00000318522}:r.1_929+805_insAA_ALK{ENST00000389048}:r.4080-115_6220	gDNA
ALK	KIF5B	KIF5B{ENST00000302418}:r.1_2183_ALK{ENST00000389048}:r.4080_6222	cDNA
ALK	KIF5B	KIF5B{ENST00000302418}:r.1_2183_ALK{ENST00000389048}:r.4094_6222	cDNA
ALK	KIF5B	KIF5B{ENST00000302418}:r.1_2183+2477_ALK{ENST00000389048}:r.4005_6222	gDNA
ALK	KIF5B	KIF5B{ENST00000302418}:r.1_2490_ALK{ENST00000389048}:r.4080_6222	cDNA

3p Gene	5p Gene	Transcript Definition	Type
ALK	KIF5B	KIF5B{ENST00000302418}:r.1_3219_ALK{ENST00000389048}:r.4080_6222	cDNA
ALK	KLC1	KLC1{ENST00000348520}:r.1_1580_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	SQSTM1	SQSTM1{ENST00000514093}:r.1_842_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	STRN	STRN{ENST00000263918}:r.1_421_ALK{ENST00000389048}:r.4080_6222	cDNA
ALK	STRN	STRN{ENST00000263918}:r.1_421+2363_ALK{ENST00000389048}:r.4080-440_6220	gDNA
ALK	STRN	STRN{ENST00000263918}:r.1_421+2483_insUGU_ALK{ENST00000389048}:r.4080-580_6220	gDNA
ALK	STRN	STRN{ENST00000263918}:r.1_421+6813_ALK{ENST00000389048}:r.4080-1756_6220	gDNA
ALK	TFG	TFG{ENST00000240851}:r.1_755+430_ALK{ENST00000389048}:r.4080-1687_6220	gDNA
ALK	TFG	TFG{ENST00000240851}:r.1_920+2559_insU_ALK{ENST00000389048}:r.4080-940_6220	gDNA
ALK	TFG	TFG{ENST00000240851}:r.1_1061_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	TFG	TFG{ENST00000240851}:r.1_755_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	TFG	TFG{ENST00000240851}:r.1_920_ALK{ENST00000389048}:r.4080_6220	cDNA
ALK	TPM3	TPM3{ENST00000368533}:r.1_717_ALK{ENST00000389048}:r.4080_6220	cDNA
CIT	FGFR2	FGFR2{ENST00000358487}:r.1_2574_CIT{ENST00000392521}:r.2961_8708	cDNA
FGFR1	BAG4	BAG4{ENST00000521282}:r.1_192_FGFR1{ENST00000397091}:r.1680_5702	cDNA
MBIP	AXL	AXL{ENST00000301178}:r.1_2767_MBIP{ENST00000416007}:r.563_1648	cDNA
NRG1	CD74	CD74{ENST00000009530}:r.1_627_NRG1{ENST00000356819}:r.963_3083	cDNA
NRG1	CD74	CD74{ENST00000009530}:r.1_882_NRG1{ENST00000356819}:r.963_3083	cDNA
NTRK1	CD74	CD74{ENST00000353334}:r.1_868_NTRK1{ENST00000392302}:r.1262_2609	cDNA
NTRK1	MPRIP	MPRIP{ENST00000395811}:r.1_3048_NTRK1{ENST00000392302}:r.1421_2609	cDNA
NTRK1	TFG	TFG{ENST00000240851}:r.1_920_NTRK1{ENST00000392302}:r.1262_2609	cDNA
NTRK1	TPM3	TPM3{ENST00000368533}:r.1_717_NTRK1{ENST00000392302}:r.1262_2609	cDNA
NTRK3	ETV6	ETV6{ENST00000396373}:r.1_1283_NTRK3{ENST00000394480}:r.1908_19984	cDNA
NTRK3	ETV6	ETV6{ENST00000396373}:r.1_737_NTRK3{ENST00000394480}:r.1719_19984	cDNA
NTRK3	ETV6	ETV6{ENST00000396373}:r.1_737_NTRK3{ENST00000394480}:r.1908_19984	cDNA
PDGFRA	SCAF11	SCAF11{ENST00000369367}:r.1_213_PDGFRA{ENST00000257290}:r.320_6576	cDNA
RET	CCDC6	CCDC6{ENST00000263102}:r.1_535+1054_RET{ENST00000355710}:r.2369-?_5659	gDNA
RET	CCDC6	CCDC6{ENST00000263102}:r.1_535_RET{ENST00000355710}:r.2369_5659	cDNA
RET	CCDC6	CCDC6{ENST00000263102}:r.1_535+1111_RET{ENST00000355710}:r.2369-807_5659	gDNA
RET	CCDC6	CCDC6{ENST00000263102}:r.1_685_RET{ENST00000355710}:r.2369_5659	cDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_2183_RET{ENST00000355710}:r.2369_5629	cDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_2372_RET{ENST00000355710}:r.2369_5629	cDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_2897_RET{ENST00000355710}:r.2369_5629	cDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_3002_RET{ENST00000355710}:r.2369_5629	cDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_3002+152_insCUUU_RET{ENST00000355710}:r.2369-12_5629	gDNA
RET	KIF5B	KIF5B{ENST00000302418}:r.1_3219_RET{ENST00000355710}:r.2112_5629	cDNA
RET	NCOA4	NCOA4{ENST00000452682}:r.1_870_RET{ENST00000355710}:r.2369_5659	cDNA
RET	TRIM33	TRIM33{ENST00000358465}:r.1_2502_RET{ENST00000355710}:r.2369_5659	cDNA
ROS1	CCDC6	CCDC6{ENST00000263102}:r.1_1079_ROS1{ENST00000368508}:r.5841_7435	cDNA
ROS1	CD74	CD74{ENST00000009530}:r.1_627_ROS1{ENST00000368508}:r.5841_7435	cDNA
ROS1	CD74	CD74{ENST00000009530}:r.1_627_ROS1{NM_002944}:r.5448_7368	cDNA
ROS1	CD74	CD74{ENST00000009530}:r.1_627_ROS1{NM_002944}:r.5757_7368	cDNA
ROS1	CLTC	CLTC{ENST00000393043}:r.1_5177_ROS1{ENST00000368508}:r.5841_7435	cDNA
ROS1	EZR	EZR{ENST00000367075}:r.1_1259_ROS1{NM_002944}:r.5757_7368	cDNA

3p Gene	5p Gene	Transcript Definition	Type
ROS1	EZR	EZR{ENST00000367075}:r.1_1259+207_oEZR{ENST00000367075}:r.1259+210_1259+244_ROS1{NM_002944}:r.5757-744_7368	gDNA
ROS1	GOPC	GOPC{ENST00000368498}:r.1_1334_ROS1{NM_002944}:r.5841_7368	cDNA
ROS1	GOPC	GOPC{ENST00000368498}:r.1_1334+2304_ROS1{NM_002944}:r.5841-2355_7368	gDNA
ROS1	GOPC	GOPC{ENST00000368498}:r.1_726_ROS1{NM_002944}:r.5977_7368	cDNA
ROS1	GOPC	GOPC{ENST00000368498}:r.1_726+822_insGAUAUGCUGAGUAUUUGCUCAAAGGAAAGUCACCUCU_ROS1{NM_002944}:r.5977-563_7368	gDNA
ROS1	LRIG3	LRIG3{ENST00000320743}:r.1_2982_ROS1{ENST00000368508}:r.5841_7435	cDNA
ROS1	SDC4	SDC4{ENST00000372733}:r.1_239_ROS1{ENST00000368508}:r.5757_7368	cDNA
ROS1	SDC4	SDC4{ENST00000372733}:r.1_239_ROS1{NM_002944}:r.5448_7368	cDNA
ROS1	SDC4	SDC4{ENST00000372733}:r.1_485_ROS1{NM_002944}:r.5448_7368	cDNA
ROS1	SDC4	SDC4{ENST00000372733}:r.1_485_ROS1{NM_002944}:r.5757_7368	cDNA
ROS1	SLC34A2	SLC34A2{ENST00000382051}:r.1_2076_ROS1{NM_002944}:r.5757_7368	cDNA
ROS1	SLC34A2	SLC34A2{ENST00000382051}:r.1_2076_ROS1{NM_002944}:r.5448_7368	cDNA
ROS1	SLC34A2	SLC34A2{ENST00000382051}:r.1_429_ROS1{NM_002944}:r.5448_7368	cDNA
ROS1	SLC34A2	SLC34A2{ENST00000382051}:r.1_429_ROS1{NM_002944}:r.5757_7368	cDNA
ROS1	TPM3	TPM3{ENST00000368530}:r.1_968_ROS1{NM_002944}:r.5841_7368	cDNA
ROS1	TPM3	TPM3{ENST00000368533}:r.1_319_ROS1{ENST00000368508}:r.5977_7435	cDNA
TACC3	FGFR3	FGFR3{ENST00000440486}:r.1_2530_TACC3{ENST00000313288}:r.1943_2781	cDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530_TACC3{ENST00000313288}:r.1751_2781	cDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530_TACC3{ENST00000313288}:r.2048_2781	cDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530+104_TACC3{ENST00000313288}:r.541_2781	cDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530+27_TACC3{ENST00000313288}:r.2048-1511_2781	cDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530+38_TACC3{ENST00000313288}:r.2048-1459_2781	gDNA
TACC3	FGFR3	FGFR3{NM_000142}:r.1_2530+63_TACC3{ENST00000313288}:r.1877_2781	cDNA

Appendix 3: Other Targets

Other Targets	Target Type
ABCB1	Expression Marker
BRCA1	Expression Marker
CD274	Expression Marker
CDKN2A	Expression Marker
CTLA4	Expression Marker
ERCC1	Expression Marker
ESR1	Expression Marker
FGFR1	Expression Marker
FGFR2	Expression Marker
IFNGR	Expression Marker
ISG15	Expression Marker
MET	Expression Marker
MSLN	Expression Marker
PDCD1	Expression Marker
PDCD1LG2	Expression Marker
PTEN	Expression Marker
RRM1	Expression Marker
TDP1	Expression Marker
TERT	Expression Marker
TLE3	Expression Marker
TOP1	Expression Marker
TUBB3	Expression Marker
TYMS	Expression Marker
ALK	3’-5’ Imbalance
NTRK1	3’-5’ Imbalance
PDGFRA	3’-5’ Imbalance
RET	3’-5’ Imbalance
ROS1	3’-5’ Imbalance
Multiple Endogenous Targets	Endogenous Controls
MET exon 14 skipping	3

Note: Claims on the Expression Markers are up to the prerogative of the client and their intended use.

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Effective Date: 20 October 2016