L1000 SOP

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Background

L1000 is a multiplexed gene expression assay that uses ligation mediated amplification (LMA) of RNA sequence specific probes combined with Luminex based detection to generate expression profiles of 978 genes per sample in a 384 well format.

Consumables

ID	Item	Location	Supplier	Cat #
C1	384 tips, 30μL	7075 RT	Nanoscreen	TIP-P30384NS-00
C2	384 tips, 15μL	7075 RT	Tecan	30051802
C3	384 tips, 50μL	7075 RT	Tecan	30051805
C4	384 tips, 125μL	7075 RT	Tecan	30051808
C5	Compression rack	7085	-	-
C6	Compression pad	7085	-	-
C7	Rack adapter	7085	-	-
C8	Storage bottle, 250mL	7075 RT	VWR	28199-758
C9	Storage bottle, 500mL	7075 RT	VWR	
C10	Reagent reservoir	7075 RT	Fisher	RES-SW1-LP
C11	Super rag	7075 RT	Blue Thunder	WI-1318Q.250
C12	Turbocapture Plate, 384 well	7082 4°	Qiagen	1057024
C13	Twintec PCR plate, 384 well	7075 RT	VWR	12000-658

Reagents

ID	Item	Location	Supplier	Cat #
R1	Nuclease free H_2O an	7075 RT	Invitrogen	AM9932
R2	DEPC H_2O	7075 RT	Broad	DEPCH2O
R3	MCF7 Total RNA	7085 -80°	Invitrogen	AM7846
R4	TCL Lysis Buffer	7085 RT	Qiagen	1057024
R5	dNTP mix, 100mM	7080 -20°	Thermo Fisher	10297-117
R6	M-MLV Buffer 5X	7080 -20°	Promega	M531X
R7	Taq DNA Ligase Buffer	7080 -20°	NEB	B0208S
R8	M-MLV Reverse Transcriptase	7080 -20°	Promega	M170X
R9	Taq DNA Ligase	7080 -20°	NEB	M0202B
R10	T7 Biotinylated Primer	7080 -20°	IDT	Custom

R11	T3 Primer	7080 -20°	IDT	Custom
R12	HotStarTaq DNA Polymerase	7080 -20°	Qiagen	203207
R13	PCR Buffer	7080 -20°	Qiagen	Included w/ enzyme
R14	$MgCl_2$	7080 -20°	Qiagen	Included w/ enzyme
R15	Epsilon probe pool	7085 -20°	IDT	Custom
R16	SAPE	7082 4°	Lifetech	STREPR-PE
R17	High stringency buffer	7082 4°	Broad	HIGHSTRINWASH
R18	Low stringency buffer	7082 4°	Broad	LOWSTRINBUF
R19	Flexmap sheath fluid	7075 RT	Broad	1XSHEATHFLUID
R20	TMAC hybridization solution	7075 RT	Broad	-
R21	Epsilon bead set	7075 4°	CMAP	-
R22	20% SSPE	7075 RT	VWR	12001
R23	12X MES Buffer	7075 RT	Broad	12XMES

Equipment

ID	Item
E1	Janus liquid handler (2)
E2	Combi liquid dispenser
E3	Agilent PlateLoc heat sealer (3)
E4	37° circulating waterbath
E5	45° circulating waterbath
E6	95° circulating waterbath
E7	Ramping waterbath (2)
E8	Agilent plate labeler
E9	Tecan liquid handler
E10	Lifetech dual block PCR cycler (25)
E11	Benchtop centrifuge (2)
E12	High capacity floor centrifuge

Mixes

Reference RNA (M1)

Reagent	Volume(μL)/well
TCL Lysis Buffer (R4)	**19.7**
MCF7 total RNA (R3)	0.3
Total (uL/well)	20

Reverse Transcription Master Mix (M2)

Reagent	Volume(μL)/well
DEPC treated H_2O (R2)	3.7
M-MLV Buffer 5X (R6)	1
dNTP mix, 100mM (R5)	0.1
M-MLV Reverse Transcriptase (R8)	0.2
Total (uL/well)	5

Probe Anneal Master Mix (M3)

Reagent	Volume(μL)/well
Nuclease free H_2O (R1)	4.288
Taq DNA Ligase Buffer (R7)	0.5
Epsilon probe pool (R15)	0.212
Total (uL/well)	5

Ligation Master Mix (M4)

Reagent	Volume(μL)/well
Nuclease free H_2O (R1)	4.4375
Taq DNA Ligase Buffer (R7)	0.5
Taq DNA Ligase (R9)	0.0625
Total (uL/well)	5

PCR Master Mix (M5)

Reagent	Volume(μL)/well
Nuclease free H_2O (R1)	12.768
PCR buffer (R13)	1.5
$MgCl_2$ (R14)	0.51
dNTP mix, 100mM (R5)	0.096
T7 biotinylated universal primer (R10)	0.015

T3 universal primer (R11)	0.015
HotstarTaq DNA Polymerase (R12)	0.096
Total (uL/well)	15

Luminex MagPlex Bead Mixture (M6)

Reagent	**Volume(μL)/well**
TMAC Hybridization Solution (R20)	30
Coupled MagPlex beads	N/A
Total (uL/well)	30

Low Stringency Wash Buffer (M7)

Reagent	**Volume(μL)/well**
DEPC treated H_2O (R2)	20.97
10% TWEEN20(R?)	0.03
20X SSPE(R22)	9
Total (uL/well)	30

High Stringency Wash Buffer (M8)

Reagent	**Volume(μL)/well**
DEPC treated H_2O (R2)	27.3
12X MES Buffer (R23)	2.5
5M NaCl	0.17
10% TWEEN20(R22)	0.03
Total (uL/well)	30

SAPE Stain Mix (M9)

Reagent	**Volume(μL)/well**
High stringency buffer (M8)	29.6
SAPE (R16)	0.4
Total (uL/well)	30

Ligation Mediated Amplification (LMA)

NOTE **Before beginning double check to ensure that:**

There are sufficient quantities of all reagents for each step
There are sufficient supplies of consumables
Work surfaces are clean and free from RNAse and DNAse

Capture

In this step lysate plates are thawed and crude cell lysate is transferred to Qiagen turbocapture plates in order to isolate cellular mRNA

1. Remove lysate plates and MCF7 total RNA from freezer and place at room temperature to thaw
2. Unbox and remove lids from appropriate number of turbocapture plates (**C12**)
3. Prepare reference RNA mixture (**M1**) using the following per-well recipe. Make enough for $\{(\boldsymbol{Number\ plates} \times \mathbf{2}) + \mathbf{20}\}$ wells. Ensure to quickly vortex and spin total RNA prior to use and keep mix on ice until ready to add

ID	Reagent	Volume
R3	MCF7 Total RNA	0.3μL
R4	TCL Lysis buffer	19.7μL

4. Remove seals from thawed lysate plates and match up with appropriate turbocapture plates (**C12**)
5. Mix and transfer 20μL of crude cell lysate from each well of the lysate plate to the turbocapture plate, ensuring that wells A1, A2, B1, and B2 are left empty
6. Add 20μL of the reference RNA mixture to wells A2 and B2 on each plate
7. Seal completed lysate plates and recap completed turbocapture plates
8. Quickly spin turbocapture plates up to 1000RPM to settle lysate and remove bubbles
9. Make note of the time that the final plate is completed. Set timer for 1 hour and leave turbocapture plates to incubate on the benchtop.
10. Place lysate plates back in the freezer
11. Take out the following reagents to thaw on ice: M-MLV buffer (**R6**), 100mM dNTPs (**R5**)

Reverse Transcription

The cell lysate is washed out of the turbocapture plates and the captured mRNA is used as a template to synthesize cDNA directly on the plate

1. Remove caps from turbocapture plates and discard to biohazard bin
2. Using Super Rags (**C11**) that have been folded twice in between each plate, load plates upside down in centrifuge
3. Prepare appropriate volume of reverse transcription master mix (**M2**) and keep on ice until ready to use

4. Spin turbocapture plates at 1000RPM for 1 minute to remove all cell lysate
5. Add 5µL master mix to each well, leaving wells A1 and B1 empty
6. Seal plates and quickly spin up to 1000RPM to settle mix and remove bubbles
7. Incubate plates at 37°C for 90 minutes in a waterbath or thermocycler
8. Remove and thaw Taq DNA Ligase buffer (**R7**) and Epsilon Probe Pool (**R15**)

Probe Anneal

The reverse transcription master mix is removed from the plates and the L1000 probe set is added in order to bind the complimentary cDNA sequences

1. Remove plates and unseal
2. Using Super Rags (**C11**) that have been cut in half and folded twice in between each plate, stack plates upside down and load in centrifuge
3. Make appropriate amount of probe anneal master mix (**M3**) and keep on ice until ready to use
4. Spin turbocapture plates at 1000RPM for 1 minute to remove all previous master mix
5. Add 5µL master mix to each well, leaving wells A1 and B1 empty
6. Seal plates and quickly spin up to 1000RPM to settle mix and remove bubbles
7. Denature plates at 95°C for 5 minutes in a waterbath or thermocycler
8. Incubate the plates in a thermocycler or waterbath, decreasing the temperature at a constant rate from 70°C to 40°C over the course of 6 hours
9. Hold plates at 4°C until ready to proceed

Ligation

The probe anneal master mix is removed from the plates and a master mix is added to ligate the two flanking proves together

1. Remove plates and unseal
2. Using Super Rags (**C11**) that have been folded twice in between each plate, load plates upside down in centrifuge
3. Make appropriate volume of ligation master mix (**M4**) and keep on ice until ready to use
4. Spin turbocapture plates at 1000RPM for 1 minute to remove all cell lysate
5. Add 5µL ligation master mix to each well, leaving wells A1 and B1 empty

6. Seal plates and quickly spin up to 1000RPM to settle mix and remove bubbles
7. Incubate plates at 45°C for 60 minutes in a waterbath or thermocycler
8. Remove and thaw T3 and T7 universal primers, 10mM dNTPs, PCR buffer, and $MgCl_2$

PCR

The ligation master mix is removed and a PCR mix is added. The ligated probes are then melted off of their target sequences and amplified

1. Remove plates and unseal
2. Using Super Rags (**C11**) that have been folded twice in between each plate, load plates upside down in centrifuge
3. Make appropriate volume of PCR master mix (**M5**) and keep on ice until ready to use
4. Spin turbocapture plates at 1000RPM for 1 minute to remove all cell lysate
5. Add 15μL ligation master mix to each well, leaving wells A1 and B1 empty
6. Seal plates and quickly spin up to 1000RPM to settle mix and remove bubbles
7. Load plates into thermocyclers and execute the following PCR protocol

Temperature (°C)	**92**	**92**	**60**	**72**	**72**	**4**
Time (mm:ss)	15:00	01:00	01:00	01:00	05:00	∞
Cycles	1		29		1	

8. Once complete, either move plates to the -20ºC freezer or proceed directly to hybridization

Hybridization

In this phase of the assay the biotinylated, uniquely barcoded PCR products will be incubated with Luminex beads containing complimentary barcode sequences

NOTE

Before beginning double check to ensure that:
1. There is enough coupled bead for all plates
2. There is sufficient POSAMP control for 5μL/plate
3. There are sufficient PCR plates

1. Remove amplicon plates from freezer and set on bench to thaw (if necessary)
2. Remove and thaw POSAMP (previously pooled, untreated MCF7 amplicon) in appropriate volumes for 5μL/plate
3. Unpack appropriate number of PCR plates, one per turbocapture plate (**C13**)
4. Add 30μL of L1000 Luminex bead hybridization mixture (**M6**) to all wells of each empty PCR plate, to be referred to as the detection plate
5. Transfer and mix 5μL amplicon from each turbocapture well to each well of the matching detection plate
6. Add 5μL POSAMP positive control to well B1 in each detection plate
7. Seal plates
8. Store remaining amplicon at -20°C
9. Denature detection plates at 95°C for 2 minutes in thermocycler or waterbath
10. Incubate detection plates at 45°C in thermocycler or waterbath for at least 16 hours

Washing and Staining

Detection plates are washed to remove any unhybridized PCR product and stained with a streptavidin-phycoerythrin (SAPE) stain. A second wash is performed to remove any unbound SAPE prior to detection.

NOTE: It is recommended to perform these wash steps using magnetic separation of Luminex beads.

Washes

1. Pellet bead by either centrifugation up to 3000RPM or using an SBS compatible magnet
2. Remove 30μL previous wash liquid or buffer and discard
3. Add 30μL next wash liquid or buffer
4. Mix up and down thoroughly
5. Pellet bead by either centrifugation up to 3000RPM or using an SBS compatible magnet
6. Remove 30μL wash liquid or buffer and discard

Preparation

1. Make up sufficient volumes of the following mixes
   a. Low stringency buffer (**M7**)
   b. High stringency buffer (**M8**)
   c. SAPE stain mix (**M9**)
   d. Luminex sheath fluid (**M10**)
   e. TMAC hybridization buffer (**M11**)

Procedure

1. Perform the following washes
    1. Low stringency buffer (**M7**)
    2. High stringency buffer (**M8**)
2. Add and mix 30μL SAPE stain mix (**M9**)
3. Incubate at room temperature for 15 minutes away from light
4. Perform the following washes
    1. Low stringency buffer
    2. Luminex sheath fluid (x3)
5. Add 30μL sheath fluid to plates and seal. Plates can be stored for up to one week at 4°C away from light sources

Detection

1. Ensure that FM3D scanner has sufficient supply of sheath fluid and sufficient room in waste output
2. Thoroughly vortex plate to be scanned
3. Ensure protocol is setup to detect all 500 analytes and aspirate the full volume of each sample well (35μL)
4. Select protocol, name plate and run (will take ~4 hours)