Use of Invitrogen reagents in conjunction with Arcturus and ABI technologies to quickly accomplish one step real-time qPCR on RNA from laser dissected cells

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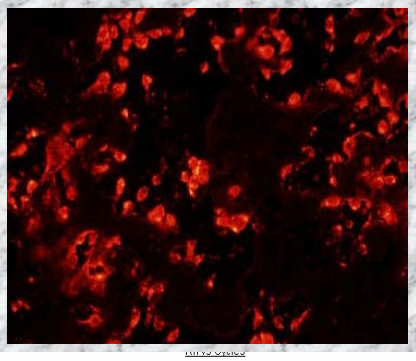
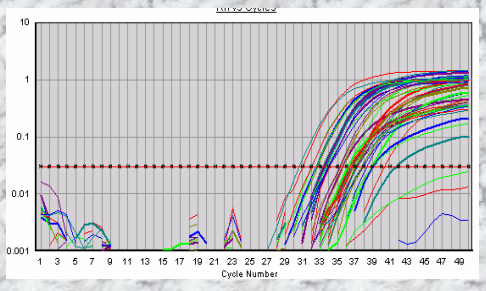
This effort (by us at ISU with key Invitrogen people) resulted in the creation of the product: CellsDirect™ One-Step RT-PCR Kit Part no. 46-7201; 500 rxns per kit.

Improved qPCR Sensitivity with Laser Capture Microdissection (LCM) Samples

- Getting quality gene expression data from small samples, particularly LCM samples, can be a challenge. The biggest issues center around RNA isolation methods, most of which (a) are not suitable for small samples, (b) result in sample loss, and (c) introduce reagents that are known to inhibit PCR. We will introduce a new technique to isolate LCM samples and perform qPCR without using RNA isolation methods, providing greater sensitivity and more reliable results.
- LCM continues to be a popular method for sample isolation, but it provides very little starting material. LCM users risk losing much of their precious samples during RNA purification, limiting their utility in qPCR. This is a major problem, which our technique overcomes.

Flowchart of Methods





Florescence staining in cryostat section

↓

Cell samples retrieved by Laser Capture Microdisection (LCM)

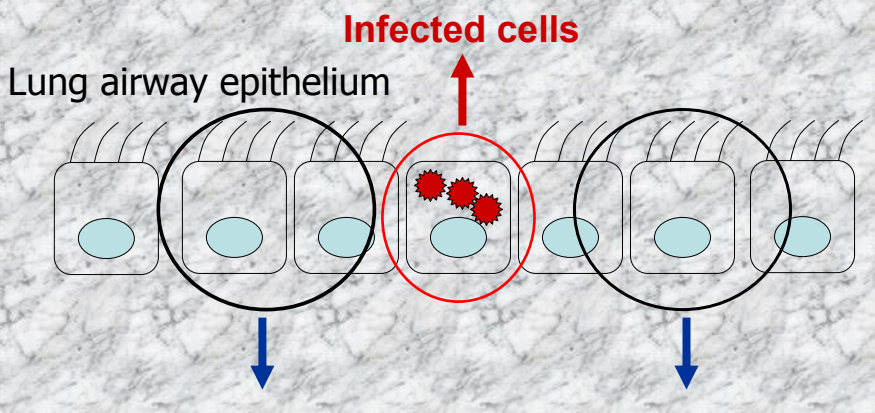
↓

RNA extraction and Isolation

↓

One step qPCR

Cells retrieved by LCM



Source of RNA isolation idea came from Sharon Lahn suggesting reading Invitrogen's CellsDirect Manual pages 2 and 4:

"…page 2:

- 11. Adjust the cell density using cold PBS so that it falls within the range of 1–10,000 cells/µl. Count the cells again to verify cell concentration.
- 12. To a 0.2-ml thin-walled PCR tube or plate well **on ice**, add 1 µl of RNaseOUT™ (40 U/µl) and 10 µl of Resuspension Buffer.
- 13. Transfer 1–2 µl of cells (<10,000 cells) to the PCR tube/well.
- **Control:** For the control reaction, add 1 µl of the Control HeLa Total RNA to the PCR tube or plate well instead of cell lysate.
- 14. Transfer the tube/plate to an incubator, water bath, or thermal cycler preheated to 75°C and incubate for 10 minutes.
- **Control:** For the control reaction, incubate for 3 minutes.
- 15. After incubation, spin briefly to collect the condensation and proceed to **DNase I Digestion,** page 4. …"

Invitrogen CellsDirect Manual p. 4

"… **DNase I Digestion**

- In this step, you treat the cell lysate with DNase I to degrade any contaminating DNA.
- 1. Place each tube/plate from Step 15, page 2, or Step 8, page 3, on ice, and add the following:

Component Amount

- DNase I, Amplification Grade (1 U/µl) 5 µl
- 10X DNase I Buffer 1.6 µl
- 2. Mix by gently pipetting up and down, and spin briefly to collect the contents.
- 3. Incubate for 5 minutes at room temperature. **Note:** A longer incubation time (up to 10 minutes) may be used for larger samples (>1,000 cells). However, incubation times exceeding 10 minutes can greatly reduce cDNA yield.
- 4. Spin briefly, and add 1.2 µl of 25 mM EDTA to each tube/well on ice. Mix by gently pipetting up and down, and spin briefly to collect the contents.
- 5. Incubate at 70°C for 5 minutes …"

Invitrogen
life technologies

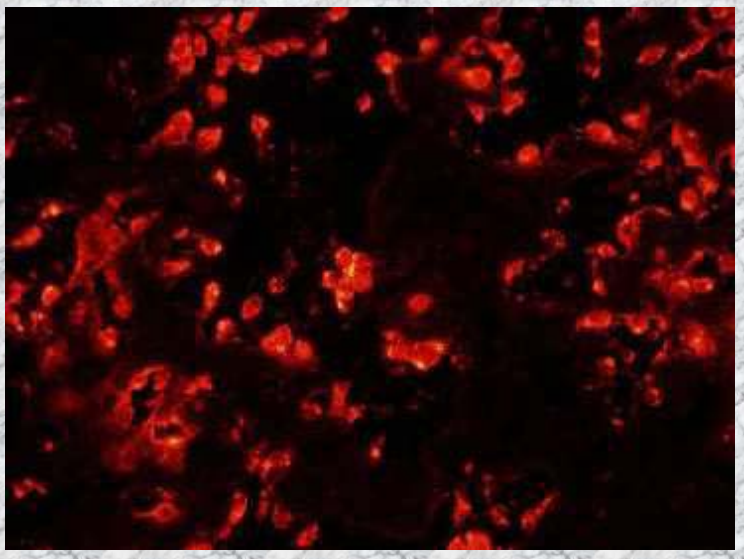
Instruction Manual

SuperScript™ III CellsDirect cDNA Synthesis System

Catalog Nos. 18080-200 and 18080-300

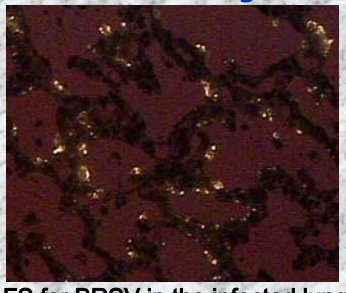
Version A
14 May 2004
25-0731

Typical frozen section on VWR superfrost slides preceding LCM



bRSV Stained by IF-IHC prior to LCM

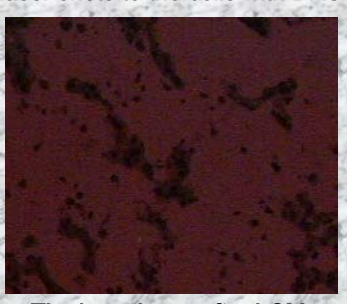
Photos for Fluorescent staining (FS) and LCM in lung tissue infected with BRSV



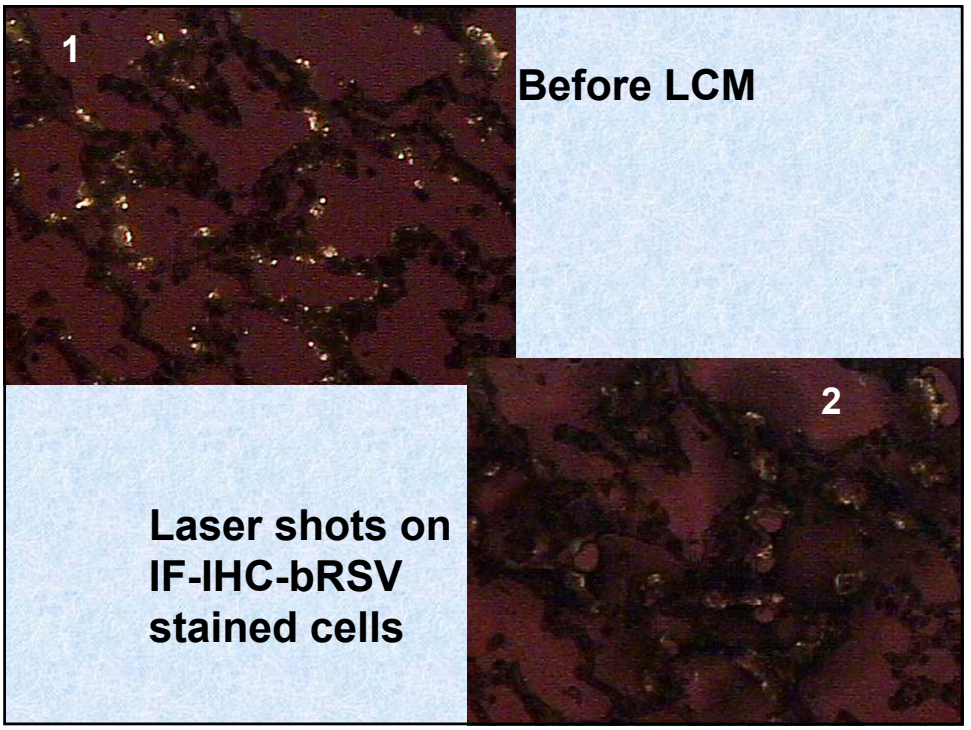
FS for BRSV in the infected lung

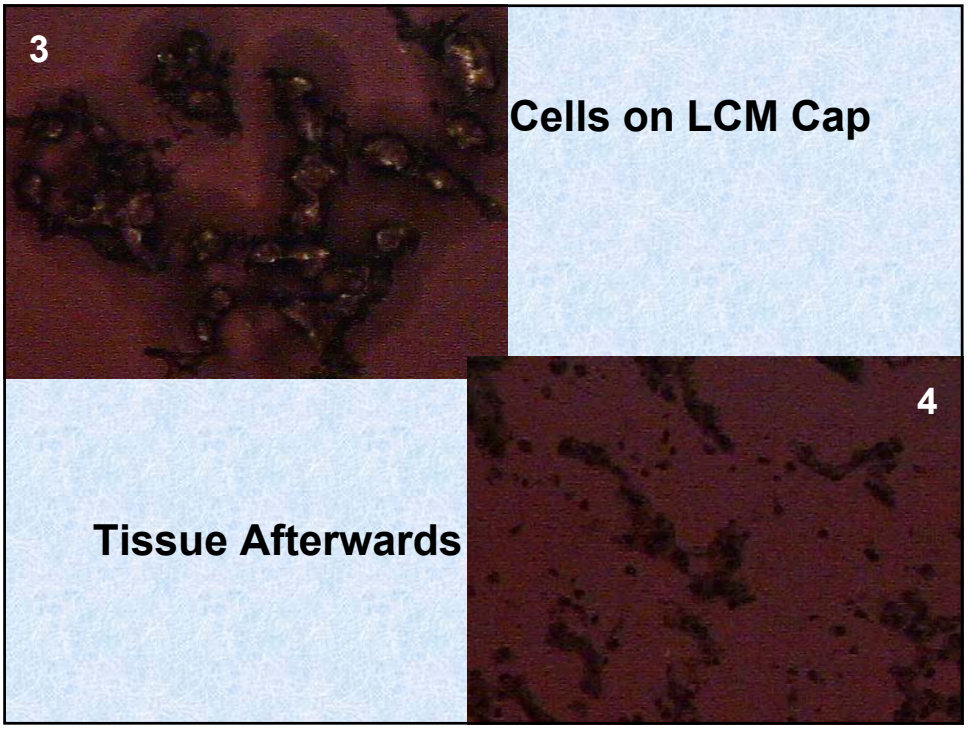
Laser shots to the cells with BRSV Ag

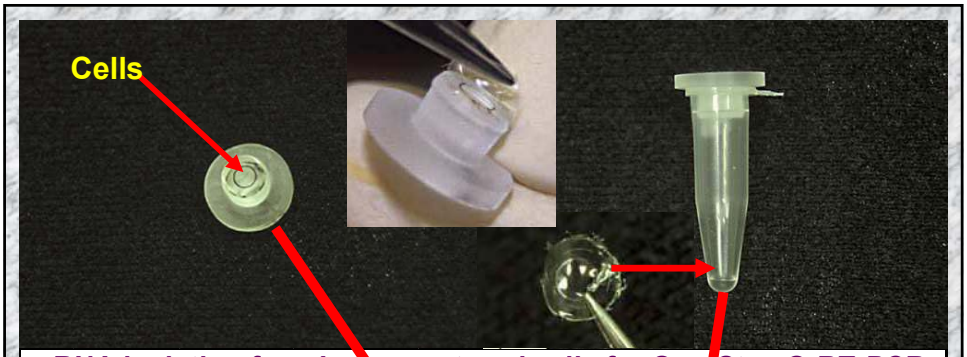
The cells captured by LCM



The lung tissue after LCM


1
Before LCM
2
Laser shots on
IF-IHC-bRSV
stained cells


3
Cells on LCM Cap
4
Tissue Afterwards



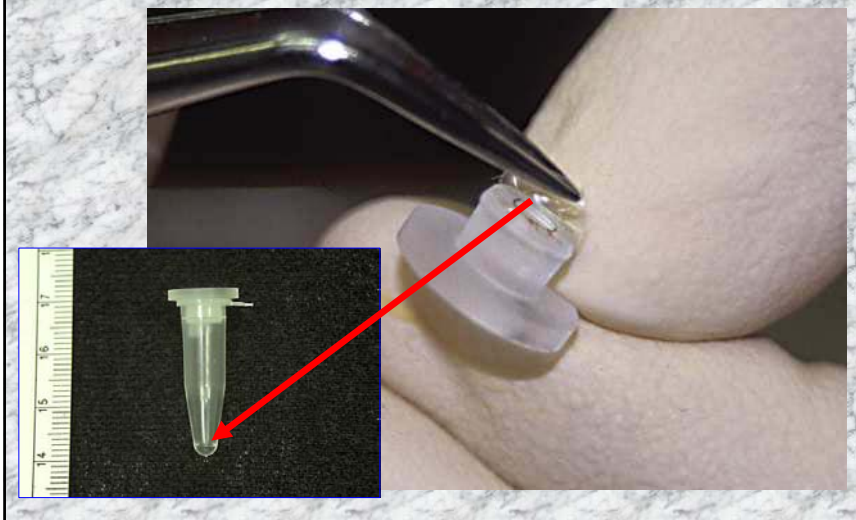
RNA isolation from Laser-captured cells for One Step Q-RT-PCR

10	caps	(1 extra sample amount already figured in)	Store at 4°C once prepared
	Resuspension Buffer:	90.9 uL	part# 55836, Invitrogen kit 11739-010
	Lysis enhancer:	10.0 uL	part# 55827, Invitrogen kit 11739-010
	new RNaseOUT:	0.1 uL	(40 U/uL) part# 55801from Invitrogen kit 18080-200
		110.00 uL	Total Lysis Buffer prepared ♦ = Lysis Buffer
			For all 10 samples
	# Cells (laser shots) per HS cap:	~25	
	Desired final sample volumes:	36 uL	
Peel cell tabs off caps and place in Lysis buffer		10 uL	Lysis Buffer used/sample

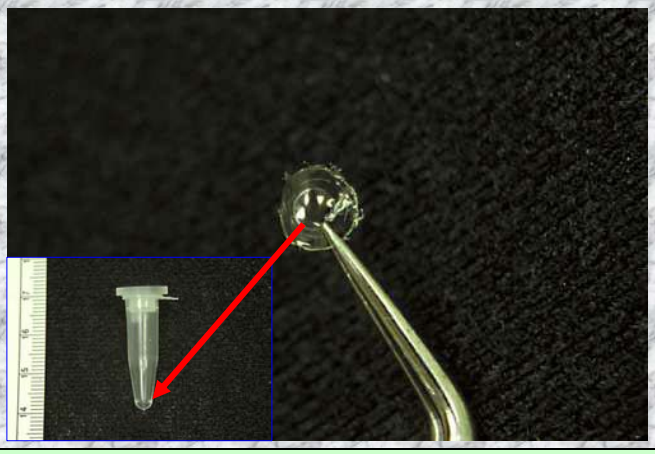
HS Caps from ARCTURUS Cat. No. LCM-0204

0.5 mL tubes: ABI Cat. No. N801-0611

Laser-captured cells on an HS LCM cap polymer tab removed from cap and shoved to the bottom of tube with a paperclip into the lysis buffer



(After capture, remove polymer tabs from HS Caps with nuclease-free forceps and put them in Lysis Buffer @ the bottom of ABI 0.5 mL tubes & incubate tubes at 50°C for 10 min. (thermo-cycler), vortex & spin samples down, pipette the liquid samples (leave tabs behind) into new tubes, then incubate at 75°C for 5 minutes to prepare samples for DNase treatment, then:



DNase Treat the Extracts:

Spin down extracts after the 75°C incubation, then to each add:

need: 16.00 uL (total needed here for all samples)

1.6 uL of 10X DNase I buffer from Cells Direct kit — part# 55805, Invitrogen kit 18080-200

and 5.0 uL of DNase I (amplification grade 1 U/uL) from CellsDirect kit — part# 55803, Invitrogen kit 18080-200

need: 50.00 uL (total needed here for all samples)

Mix gently by vortexing, microfuge briefly to collect contents, incubate at room temperature for no longer than 5 minutes, then:

Glycogen use - Optional

Vortex gently, microfuge briefly, incubate @ 75°C for 15 min. (thermocycler), then put on ice for the duration of the protocol if running real-time plates right away -- to keep transcripts linear,

Store at 4°C once prepared

Mix 198.2 uL nuclease-free H_2O + 15.2 uL 20 ug/uL glycogen solution, and add: 19.4 uL of this mix to each sample, vortex and spin down,

This is Ultrapure Glycogen from Invitrogen, Cat. No. 10814-010

1.4274 ug/uL glycogen sol'n prepared

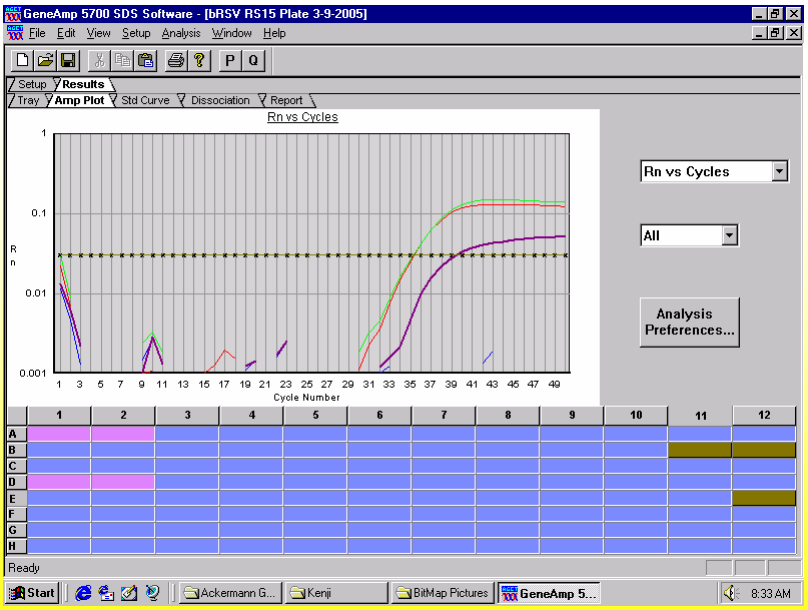
Then: *transfer each sample to new 0.2 mL tubes and store @ -80°C. Or use now.

Final Sample Volumes for one step qPCR

When using -80°C RNA samples, these 0.2 mL tubes are incubated in a thermocycler at 65°C for 5 minutes then put on ice for 1 minute just prior to being used as templates for One-Step real-time RT-PCR

OK		Each sample is now:	36 uL	containing:	0.769	ug/uL glycogen
	and:	~0.69	cells worth of RNA/uL from		25	cells/cap sample(s) …
thus:	7.80 uL	contains about:	5.42	cells worth of RNA; the suggested minimum RNA/well is RNA from 10-20 cells		
	7.8 uL of each RNA sample ends up in a final reaction volume of:				30 uL	for real-time LCM-RT-PCR
	~10	pg RNA/cell thus	7.80 uL	RNA sample should contain roughly:	54	pg RNA/each real-time rxn well
	Targets to be investigated:		Targets?	Housekeepers?	~1.81 pg/uL in each reaction well	

Signal from 1 cell's worth of RNA



Acknowledgements:

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