PROTOCOLS FOR ANALYSIS OF WHOLE SALIVA GENE EXPRESSION

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Context

- Saliva is a convenient biospecimen source
  - field, trial and population based studies
  - collected for protein, hormone and metabolic studies
- Salivary biomarkers have been identified
  - Oral squamous cell carcinoma
  - Sleep deprivation
  - Oral cavity metagenomics and disease
- Field has progressed through several approaches to collecting oral cavity biospecimens
  - Collection kits for saliva storage and future DNA and RNA extraction are commercially available

Chronic Stress and Gene Expression

- Chronic stress a risk factor
  - substance use/dependence and cancer progression
- Cole & Miller identified a consistent gene expression signature of chronic stress in lymphocytes:
  - Gene expression signature
    - Decreased glucocorticoid receptor regulated gene expression
    - Increased NF-κB regulated gene expression
  - Samples
    - 28 individuals from top and bottom 15% loneliness scale, 2007
    - 11 familial caregivers of brain cancer patients and 10 controls, 2008
    - 60 individuals stratified on early life SES, 2009
- Goal: validate stress gene expression signature in whole saliva from young adults stratified on chronic stress

Oregon Youth Substance Use Project

- Fifteen-year ongoing longitudinal study examining the etiology of substance use, including nicotine dependence
- Participants were recruited from a single school district in a working class community in Western Oregon, using a stratified random sample
- Parental consent for 1075 students (50.7% response rate)
- OYSUP sample similar to Oregon population
  - 68.8% male, 91.7% white, 4.2% Native American and 4.2% were Hispanic
  - 44% of the sample were eligible for free and reduced lunch
  - 17% of mothers and 30% of fathers did not attain an education past high school
- Participants were on average 21.05 years of age (SD=0.38).

Life Events and Difficulty Schedule

- Contextually based assessment of participants' episodic and chronic life events and difficulties
- Captures a multitude of developmental challenges encountered
  - entering or struggling with college, problems finding a job, having children, moving out of their childhood homes, developing and maintaining romantic and platonic relationships, and achieving financial self-sufficiency.
- Semi-structured interview that identifies 1) discrete events and 2) chronic difficulties in 10 life domains
  - finances, work, education, housing, health, reproduction, crime/legal, and relationships (partner and others).
- Immediate and long-term severities or threats were evaluated
- Raters who were blind to the interviewee's subjective responses assign standardized ratings to events and difficulties from cases presented by the interviewer.

Stratified by LEDS

- 48 participants from the first 123 individuals available for evaluation were selected
- 24 (6 F, 18 M) of 123 individuals experienced the “lowest” level of stressors and were chosen as the low stress stratum sample.
- 31 of 123 experienced the “highest” level of stressors – an ongoing (N=26) or recent (within the last four months, N=5) difficulty rated high in threat. We chose all available males (N=16) and randomly selected 8 of 15 available females to comprise the high stress stratum.

Participant Characteristics

Characteristic	High Stress	Low Stress	Two-sided Fisher P
	24 (50)	24 (50)	
Male Gender	16 (67)	18 (75)	0.75
Self-identified Race			
White	23 (96)	24 (100)	
Native American	1 (4)	0 (0)	>0.99
Self-identified Ethnicity			
Hispanic	2 (8)	1 (4)	
Not Hispanic	22 (92)	23 (96)	>0.99
Smoking			
Current	10 (42)	1 (4)	
Former	2 (8)	4 (17)	
Never	12 (50)	19 (79)	0.02

Total RNA Extraction

- Total RNA (excluding small RNAs) was extracted from 48 participants
  - 1ml starting volume
    - 500ul saliva + 500ul Oragene Stabilization Reagent (DNA Genotek)
  - Oragene•RNA extraction protocol with RNeasy Mini Kit and RNase-Free DNase (Qiagen)
    - incubation at 90◦C for 15 minutes
    - addition of a neutralizer solution
    - ethanol precipitation
    - on-column DNase digestion
    - column purification
  - A second DNAse treatment was also performed using TURBO™ DNA-free (Ambion) to ensure digestion of contaminating DNA

Saliva and Salivary RNA Metrics

		Saliva	Nanodrop				Agilent Analyzer		
		Volume	**Yield**	**Conc**	**260/280**	**260/230**	**RIN**	**AUC**	**Conc**	**28S/18S**
Low Stress Stratum	Mean	4.6	4.26	177.48	1.99	1.63	7.50	145.01	83.50	1.28
	SD	0.6	2.39	99.65	0.05	0.46	1.96	101.67	59.75	1.08
	Median	4.5	3.79	158.03	2.00	1.76	7.90	119.60	66.50	1.00
	Minimum	3.5	1.07	44.42	1.80	0.16	2.10	21.40	20.00	0.00
	Maximum	6.0	9.88	411.82	2.05	2.06	9.80	451.30	230.00	6.10
High Stress Stratum	Mean	4.5	4.59	191.16	1.96	1.51	6.02	157.90	84.08	0.83
	SD	0.5	5.23	217.78	0.06	0.36	2.78	227.41	114.47	0.60
	Median	4.5	2.44	101.56	1.99	1.55	7.00	53.15	34.00	1.00
	Minimum	3.7	0.73	30.46	1.76	0.72	1.00	2.30	2.00	0.00
	Maximum	6.0	19.69	820.23	2.02	2.03	9.60	823.10	412.00	2.20
	P	0.81	0.78	0.78	0.10	0.25	**0.04**	0.80	0.98	0.09

Gene Selection

- 38 test genes previously defined as over (18 genes) and under (20 genes) expressed in high versus low stress individuals:
  - Miller et al, 2008 (PMID: 18440494)
  - Higher frequency of NFκB and GR TFBMs
  - Increased fold-change expression ratios for NFκB and GR regulated genes
- Reference genes
  - 5 from Vandesompele et al, 2002 (PMID: 12184808)
  - 1 from Noutsias et al, 2008 (PMID: 18194512)

Gene expression assay selection

- ABI TaqMan® gene expression assays were chosen based on the following criteria:
  - assays with probes that span an exon junction and that would not detect genomic DNA
  - assay at or near the 3’ end of the gene
  - highest possible number of RefSeq or GeneBank entries
  - shortest possible amplicon length, and
  - inventoried

RT, Pre-Amp and qPCR

- RT reactions were performed with
  - negative controls lacking reverse transcriptase (–RT)
  - with no template (NTC)
- Multiplex pre-amplification reaction of all TaqMan® gene expression assays
- qPCR performed on Fluidigm BioMark™ system
  - 6*2304 reaction well chips contained, in triplicate,
  - 4 high stress/4 low stress RNA samples, reference RNA sample, -RT pooled saliva, NTC, and
  - a 5 point serial dilution curve in triplicate made from pooled saliva cDNA was included on each chip to normalize Ct values across chips and perform a standard curve analysis

Performance of Selected TaqMan® Gene Expression Assays

Gene	Intercept	Slope	R^2	Correlation	Efficiency	SD	CV
GADD45B	17.14	-3.34	0.999	-1	0.993	0.015	0.016
GALC	24.47	-4.00	0.993	-0.996	0.778	0.042	0.053
GBP1	18.94	-3.56	0.999	-1	0.909	0.020	0.022
HSPA1B	16.55	-3.65	0.999	-1	0.879	0.012	0.014
IL8	14.34	-3.65	1	-1	0.881	0.005	0.006
NSF	23.60	-3.99	0.999	-0.999	0.780	0.048	0.062
RAB27A	21.68	-3.71	0.998	-0.999	0.86	0.044	0.051
SLC35A1	24.20	-3.54	0.998	-0.999	0.915	0.087	0.079
STX7	20.59	-3.39	1	-1	0.972	0.030	0.030

Differentially Expressed Assays

	CSC				RSC			
Assay	**M(SD) [H]**	**M (SD) [L]**	***t***	***P***	**M (SD) [H]**	**M (SD) [L]**	***t***	***P***
GADD45B	0.85 (0.04)	0.87 (0.04)	1.19	0.242	1.10 (0.85)	0.64 (0.33)	-2.40	0.023
GALC	1.33 (0.09)	1.25 (0.06)	-2.98	0.007	0.52 (0.41)	0.88 (0.48)	2.48	0.018
GBP1	1.05 (0.11)	0.98 (0.1)	-2.22	0.032	0.45 (0.64)	0.78 (0.75)	1.43	0.161
HSPA1B	0.79 (0.06)	0.80 (0.05)	0.89	0.379	1.60 (1.59)	0.80 (0.44)	-2.37	0.026
IL8	0.84 (0.18)	0.70 (0.11)	-3.37	0.002	0.24 (0.24)	0.55 (0.36)	3.46	0.001
NSF	1.25 (0.06)	1.21 (0.04)	-2.03	0.049	0.53 (0.27)	0.70 (0.23)	2.18	0.036
RAB27A	1.17 (0.06)	1.13 (0.03)	-2.51	0.019	0.41 (0.24)	0.57 (0.21)	2.31	0.026
SLC35A1	1.31 (0.07)	1.27 (0.04)	-1.98	0.060	0.56 (0.29)	0.77 (0.29)	2.21	0.034
STX7	1.11 (0.05)	1.08 (0.03)	-2.15	0.038	0.41 (0.25)	0.57 (0.27)	2.11	0.041

Salivary RNA and Clinical Covariates

	CSC				RSC			
Gene	**Yield**	**Gender**	**Stress**	**Smoking**	**Yield**	**Gender**	**Stress**	**Smoking**
FGL2	4.22***	-0.9	0.23	0.37	-3.64***	1.73	-0.25	-0.33
GADD45B	0.66	-1.17	-0.24	-1.35	0.51	0.82	1.39	2.26*
GALC	0.29	-0.2	1.98	0.8	-1.21	-0.01	-1.51	-0.17
GBP1	1.75	-0.28	0.91	0.49	-0.93	0.33	-0.6	-0.58
HSPA1B	0.95	-1.41	-0.12	0.11	1.51	0.47	1.14	2.05*
IL8	2.63*	-2.88**	2.48**	2.97**	-1.64	2.22*	-2.61*	-1.1
IL8[1]	2.59*	-2.63*	2.45*	2.93**	-1.56	2.25*	-2.55*	-1.08
NSF	-2.05*	0.62	0.24	0.76	1.13	0.36	-0.42	-0.49
RAB27A	2.33*	0.11	1.89	0.41	-1.54	-0.05	-1.42	-0.46
SLC35A1	-1.04	1.5	0.92	1.22	-0.91	0.38	-1.02	-0.74
STX7	1.2	-0.67	1.57	1.19	-2.66*	1.51	-1.26	-1.11

RNA extractions for genome-wide gene expression analyses

- We extracted total RNA and small RNA as follows:
  - Extraction Method for Expression Analysis
    - Selected 8 Low Stress never smokers, 8 High Stress smokers, 8 High Stress never smokers
    - Same extraction method used for previous qPCR from 1ml volume using RNEASY kit with added DNAse treatment
    - Half the samples had ExpressArt® NucleoGuard, a nuclease inhibitor, added to the RLT buffer step
  - Extraction Method for Wafergen
    - Selected 4 Low Stress and 4 High Stress never smokers
    - 500µl aliquot at 90°C for 15 min and let cool, then follow *mir*Vana™ miRNA Isolation Kit protocol starting with organic extraction.

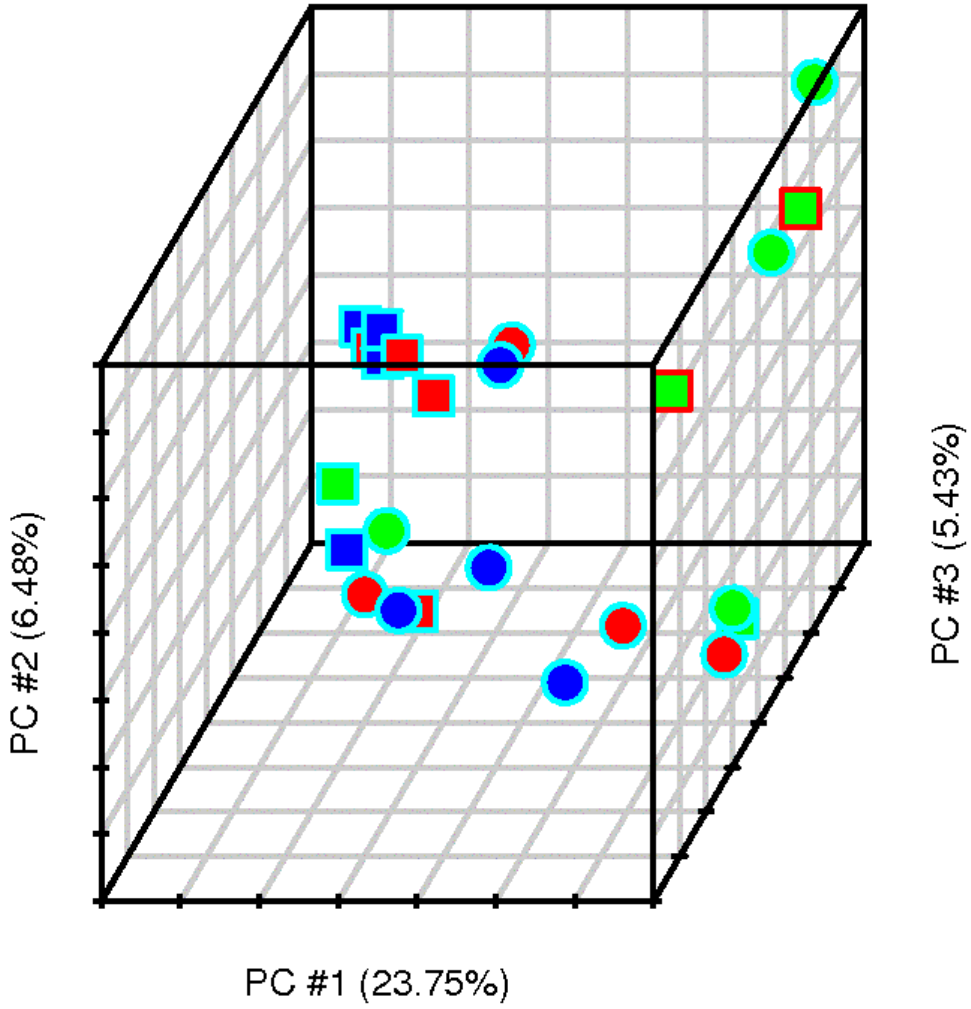
Illumina Direct Hyb HumanHT-12 v4

- 5µl total RNA (approx 200-500ng) converted into labeled target cRNA using the Illumina TotalPrep-96 RNA Amplification Kit
- 750 ng of purified biotinylated cRNA was added to Hybridization Cocktail Buffer (Illumina), applied to arrays, and incubated at 58°C for 16 hours
- Following hybridization, arrays were washed and stained using standard Illumina procedures for washing, staining and scanning
- Scanned images were processed by the Gene Expression module of GenomeStudio (v 1.6, Illumina) using default parameters without normalization

Illumina Direct Hyb data analysis

- Raw data was background corrected using “model-based background correction" methodology [PMID: 18450815]. This methods utilizes negative control probes along with test probes to estimate the unobservable background intensities using the normal exponential deconvolution model.
- The log2-transformed background subtracted data was further normalized to correct for between sample variation using the quantile normalization method [PMID: 12538238].
- We performed PCA on background corrected normalized data for all probes and all samples as implemented, and a one-way analysis of variance (ANOVA) implemented, including a cohort effect, where samples were divided in to three cohorts: (A) High-Stress-Smokers, (B) High-Stress-nonSmokers, and (C) Low-Stress-nonSmokers.
- We performed unsupervised hierarchical clustering of 38 probes with ANOVA $P<0.001$ from all 24 samples.

PCA of all 24 samples and 47K probes



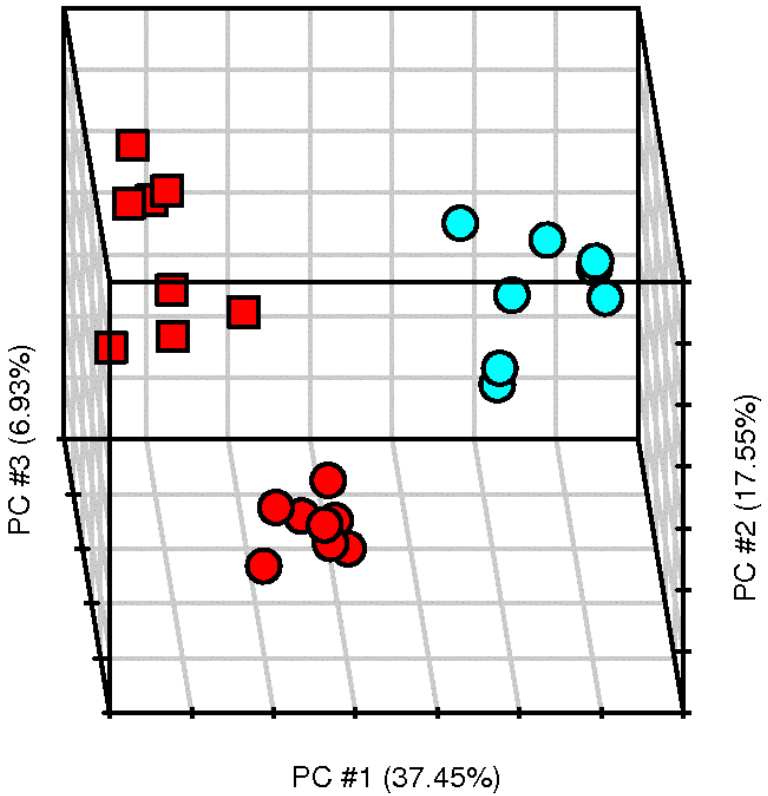
Cohort
- High Stress, Non–Smoker
- High Stress, Smoker
- Low Stress, Non–Smoker

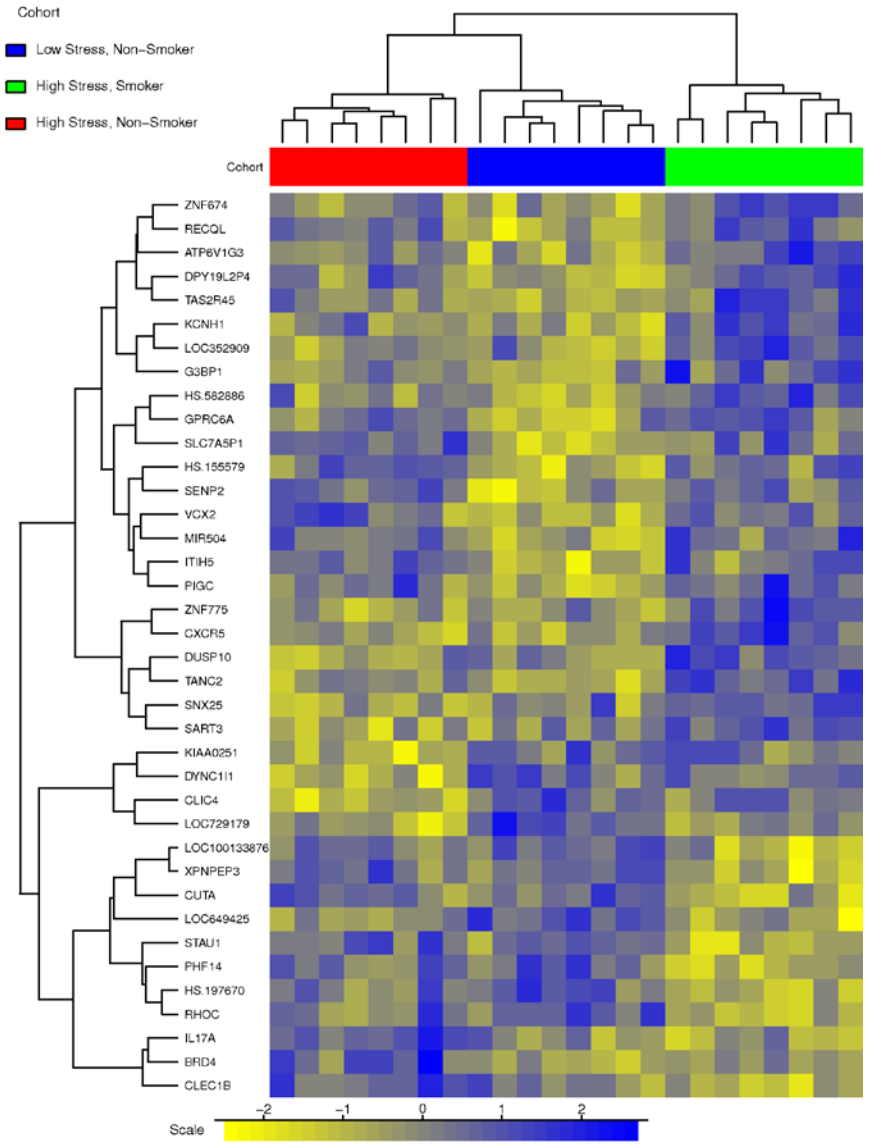
Gender
- female
- male

DateExtracted
- 1/5/2011
- 1/6/2011

PCA using all probes and all samples. Slight separation among smokers.
No batch effects, no clustering by processing date, gender or ethnicity.

PCA of all 24 samples and 38 probes





PCA and unsupervised hierarchical clustering using differentially expressed probes ANOVA *P*<0.001

qPCR versus Illumina Results

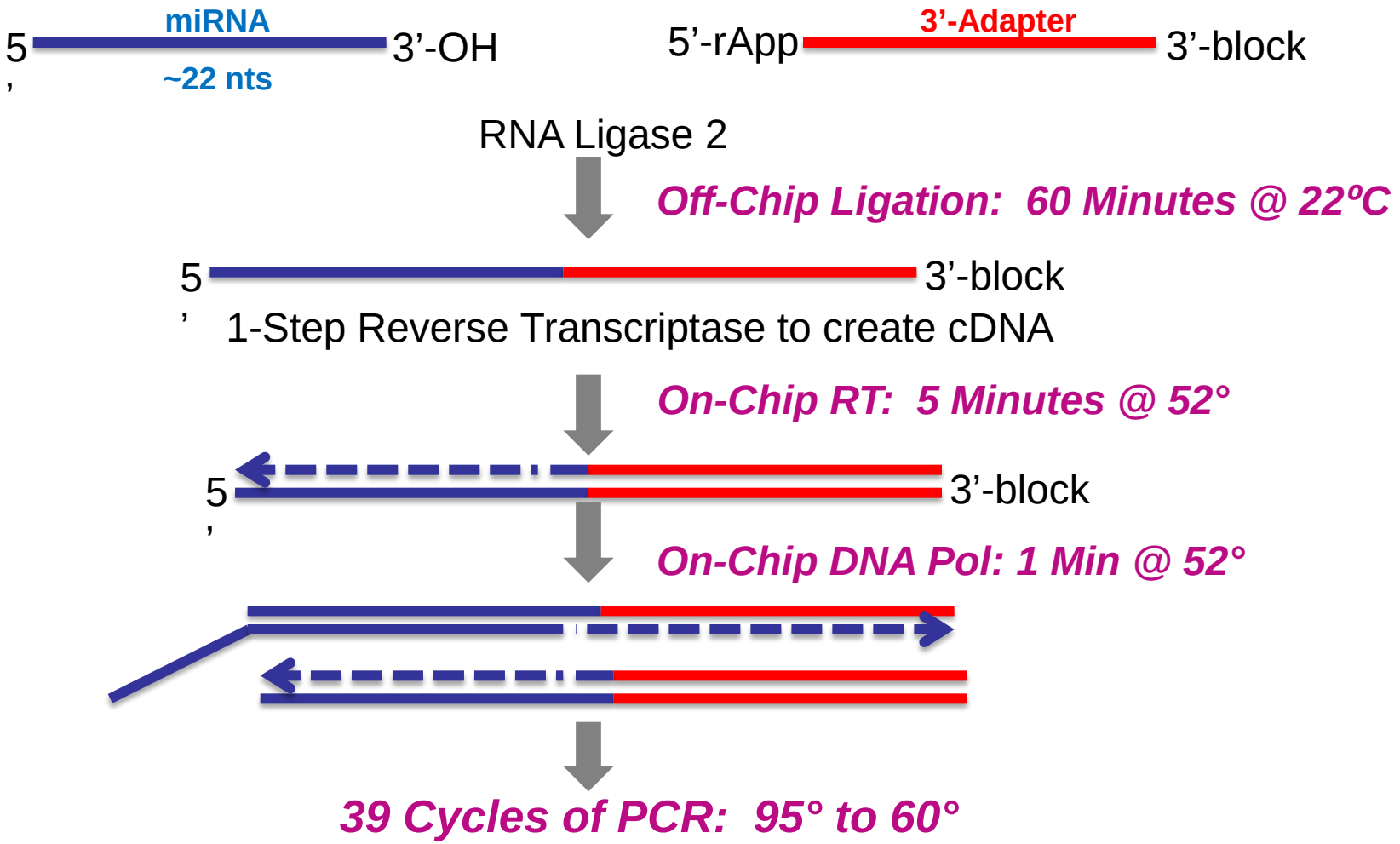
Gene	qPCR CSC *t*	qPCR CSC *P*	FC Illumina	*P* Value
GADD45B	-2.40*	0.023	-1.55	0.076
GALC	-2.98	0.007	1.27	0.210
GBP1	-2.22	0.032	1.06	0.504
HSPA1B	-2.37*	0.026	-1.16	0.450
IL8	-3.37	0.002	-9.56	0.019
NSF	-2.03	0.049	-1.08	0.659
RAB27A	-2.51	0.019	1.06	0.723
SLC35A1	-1.98	0.060	1.01	0.934
STX7	-2.15	0.038	1.07	0.545

*RSC *t* and *P*

Wafergen SmartChip miRNA panel

- 8 RNA samples (4 high and 4 low stress)
- 1 µg RNA per chip
- 1176 micro RNA assays in quadruplicate
- Targets that pass the QC analysis are designated “informative”
- Samples with informative targets are analyzed for differential expression with comparative Ct analysis
- Normalization uses all informative miR assays

Outline of Protocol



Preliminary miRome-wide data

Chip ID	Sample	Informative miRs
31851	SRI Sample 29353-310-6 miRNA V2	385
31902	SRI Sample 29360-355-6 miRNA V2	370
31818	SRI Sample 29352-309-6 miRNA V2	453
31839	SRI Sample 29347-286-6 miRNA V2	507
31814	SRI Sample 29349-289-6 miRNA V2	557
31909	SRI Sample 29348-288-6 miRNA V2	838
31911	SRI Sample 29343-262-6 miRNA V2	424
33492	SRI Sample 29341-257-6 miRNA V2	454
31892	Positive Blood	655

Summary I

- We are using whole saliva as a biospecimen source of for both trait (germline DNA) and state (mRNA and miR gene expression) analyses related to stress and smoking status.
- We have extracted RNA using a variety of methods from whole saliva and observe significant differences in salivary metrics in:
  - the RIN score between stress strata
  - the 28S/18S ratio between methods differing by the use of a RNase inhibitor,
  - multiple salivary RNA metrics between methods differing by the use of DNase
- We analyzed gene expression via qPCR in salivary RNA from 48 individuals and 37 selected candidate genes and observed:
  - excellent assay performance from TaqMan® gene expression assays
  - validated a previously described gene expression signature
  - identified a differentially expressed gene robust to multiple covariate regression.

Summary II

- We have analyzed genome-wide mRNA and miR expression using Ilumina Direct Hyb HumanHT12 v4 and Wafergen SmartChip miR panels
- Both gene expression collaborators observed lower expression than in “normal” tissues
- Both expression panels identified differentially expressed genes
- We confirmed significant differential *IL8* gene expression in the Illumina panel

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