イルミナウェビナー RNA-Seq をはじめよう！シリーズ：

0.1 pg の mRNA をシーケンスする高精度な RNA-Seq法: Quartz-Seq

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バイオインフォマティクス研究開発ユニット
http://bit.accc.riken.jp/

バイオインフォマティクス研究開発ユニット

インフォマティクスから始まるライフサイエンス

二階堂愛

(ユニットリーダー)

ソフトウェア開発

データサイエンス

$$\dot{x}_i \equiv \frac{x_i(t+\Delta t) - x_i(t)}{\Delta t} = -\eta_i x_i(t) + f_i + \sqrt{\frac{D_i}{\Delta t}}\xi_i(t)$$

インフォマティクス

分子生物学

笹川洋平

(上級研究員)

シーケンス技術開発

ライブラリ作成技術

シーケンスのデータ解析手法・ソフトウェアと実験の開発

実験生物学者へのシーケンス技術の支援

理研共通データ解析プラットフォームの構築

本日のメニュー

0.1 pg の mRNA をシーケンスする高精度なRNA-Seq法: Quartz-Seq

0. なぜ1細胞RNA-Seqが必要か?

1. 1細胞RNA-Seq: Quartz-Seqの原理と利点

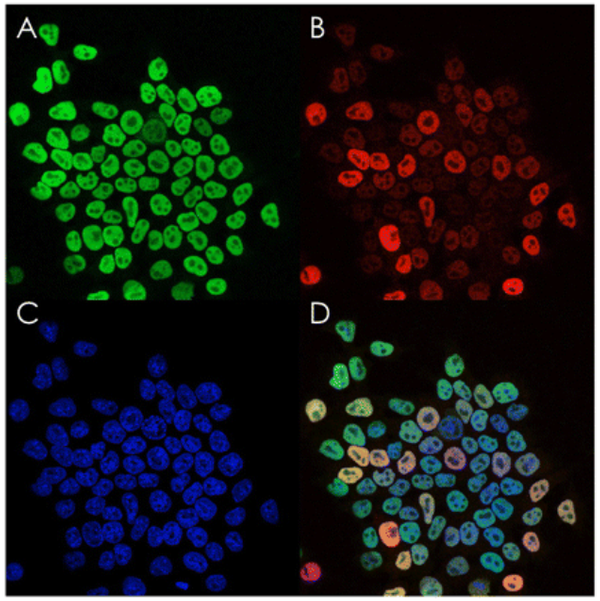
2. Quartz-Seqの性能と他の手法との比較

3. Quartz-Seqの導入方法と注意点

Cellular heterogeneity

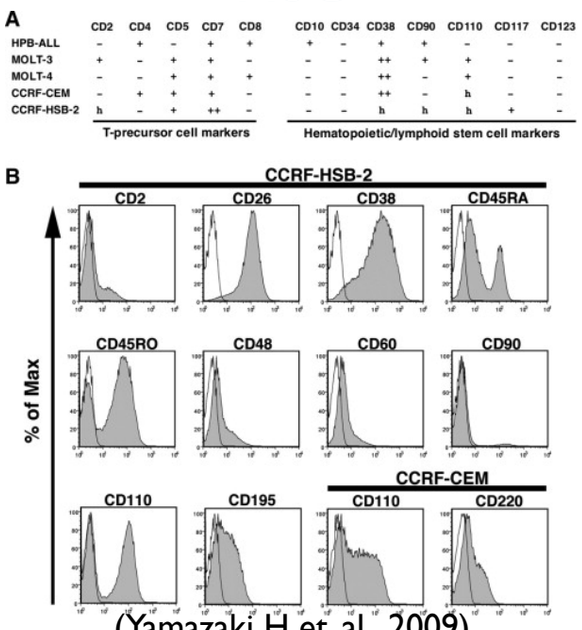
Biological role of gene expression variability

ESC



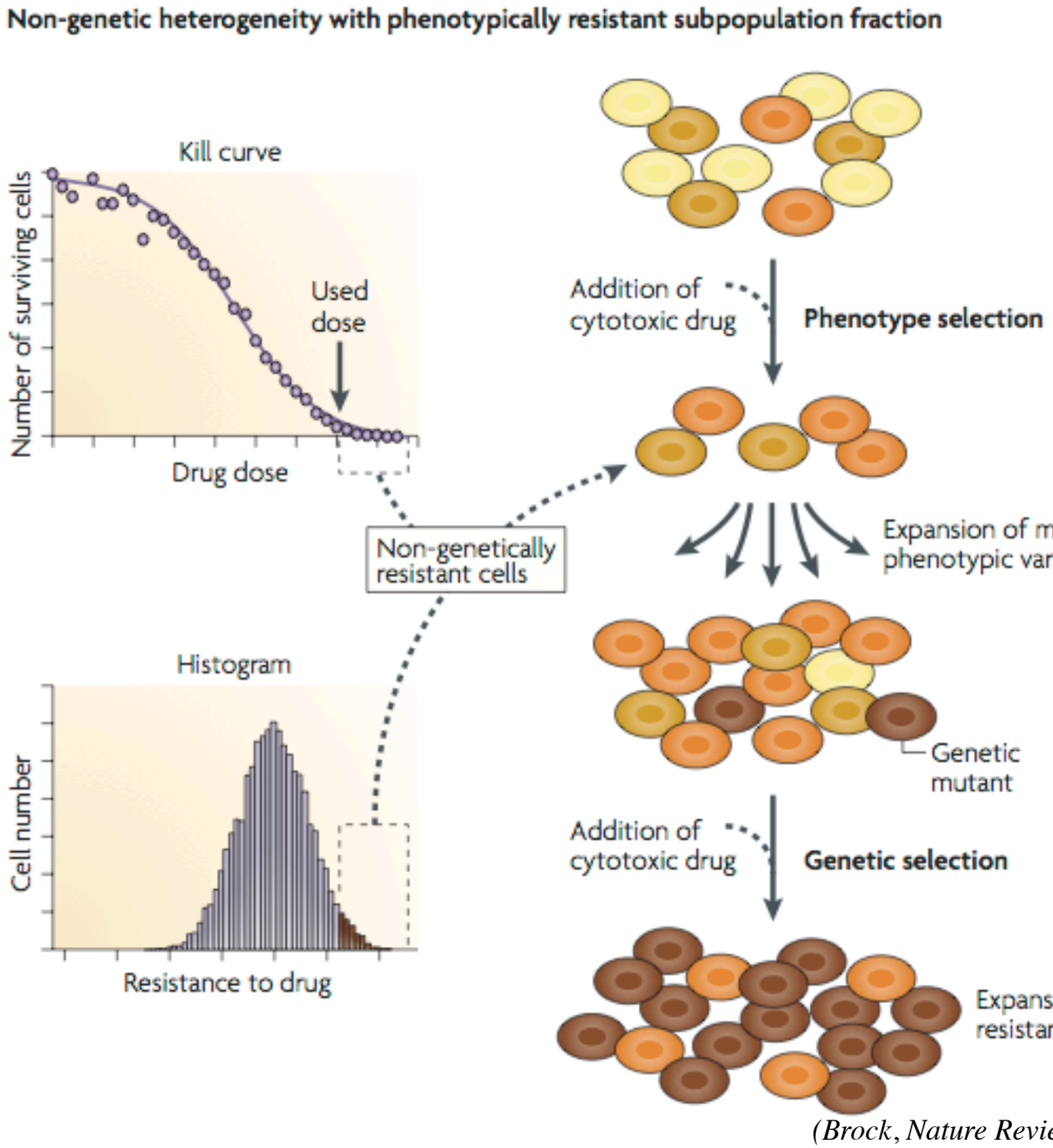
(Roeder I et. al. 2009)

HSC



(Yamazaki H et. al. 2009)

Non-genetic population evolution of drug-resistant clones in cancer progression



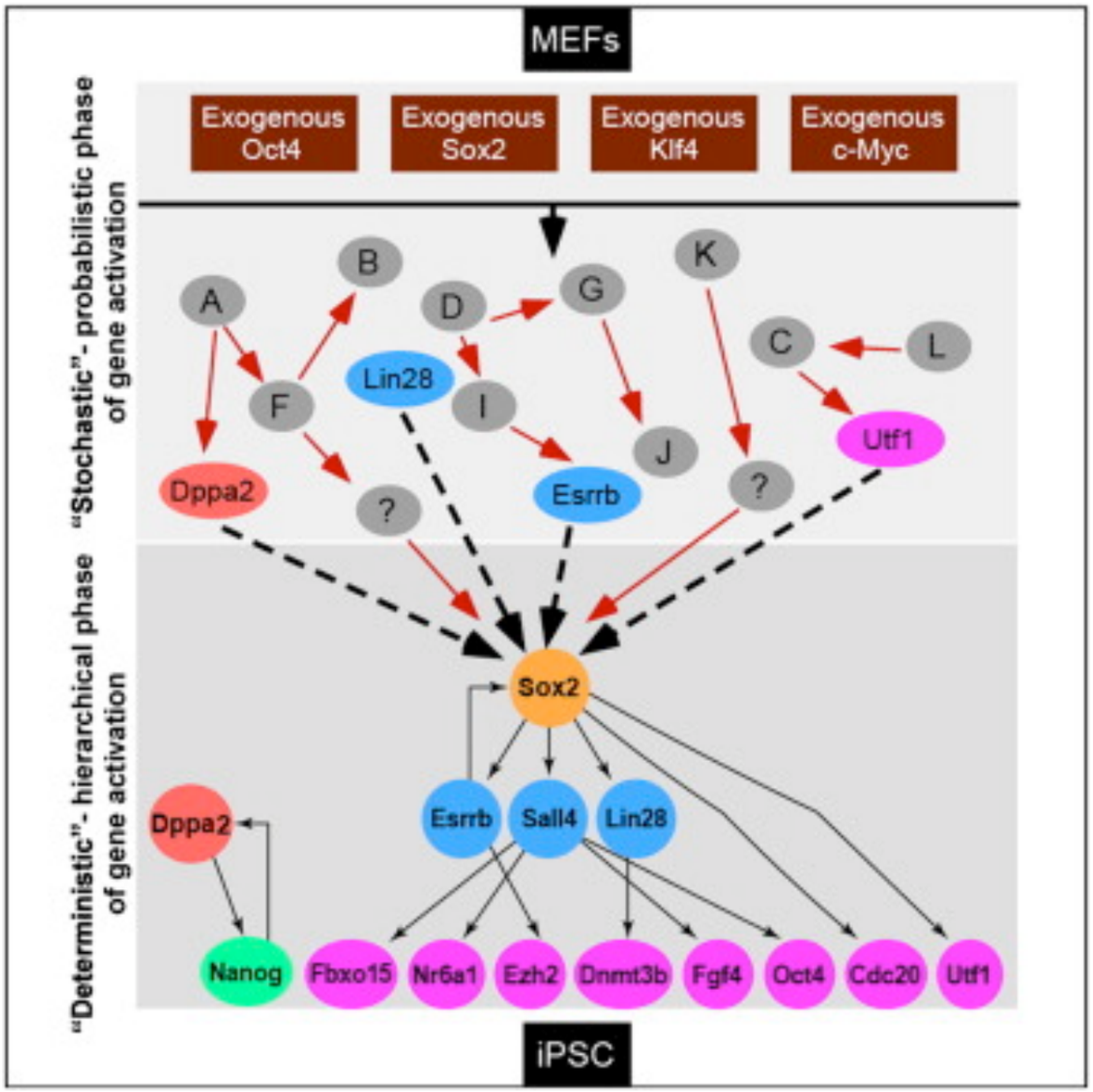
(Brock, Nature Reviews Genetics , 2009)

Cellular heterogeneity

Biological role of gene expression variability

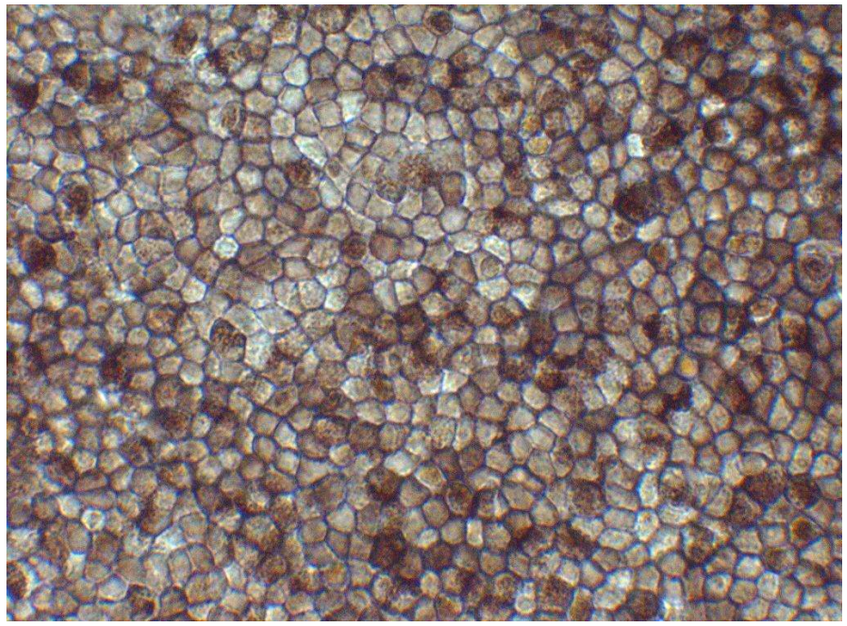
iPSの確率的なリプログラミング

1細胞qPCR (Bugaim Y et. al. Cell. 2012)



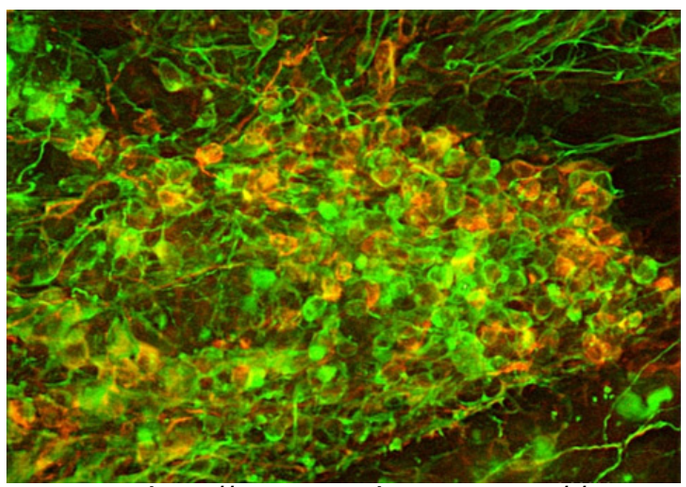
iPSの安全性評価と標準化

網膜色素上皮細胞



http://www.cdb.riken.jp/jp/

ドーパミン放出細胞



http://www.cira.kyoto-u.ac.jp/j/research/takahashi_summary.html

ゆらぎはどこからくるのか?

Source of cell heterogeneity

ゆらぎを正確に捉える技術が必要

Highly reproducible and sensitive detection method of gene expression variability

Single-Cell Genomics

Biological role of gene expression variability

Grants / Center

US NIH: US$90 million over five years
http://www.nature.com/nature/journal/v480/n7375/full/480133a.html#/bx1

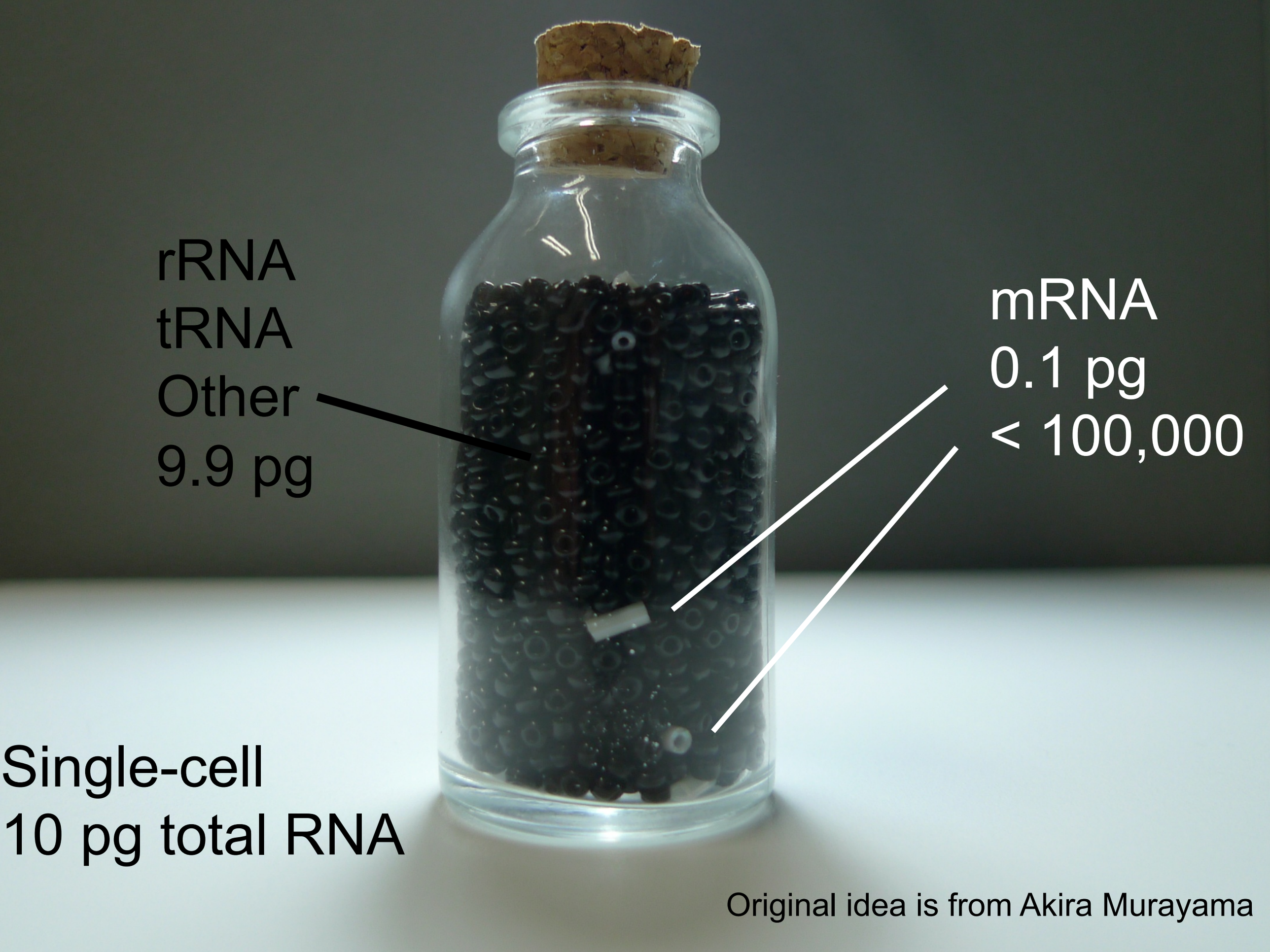
Single-cell genomics centre launched in Broad institute
http://blogs.nature.com/news/2012/05/single-cell-genomics-center-launched.html

Meeting (2013)

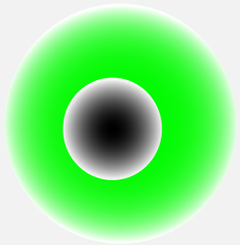
The 7th International Workshop on Approaches to Single-Cell Analysis
http://singlecellanalysis.stanford.edu
Single-Cell Genomics
http://www.weizmann.ac.il/conferences/SCG/welcome
Single-Cell Sequencing Conference
http://www.healthtech.com/Conferences_Overview.aspx?id=123234

Group

RIKEN CLUT (Single-Cell Sequencer)
Fluidigm Corporation (BioMark + C1 + Smart-Seq)
Karolinska Institutet (STRT-Seq, Smart-Seq)
Azim Surani, Gurdon Insititute


rRNA
tRNA
Other
9.9 pg
mRNA
0.1 pg
< 100,000
Single-cell
10 pg total RNA
Original idea is from Akira Murayama

Whole transcript amplification is essential for comprehensive expression profiling from single-cell



Single cell

10pg Total RNA

1%

mRNA

5’ AAAAAAAAAA 3’

100,000 molecules

(If average length of mRNA is 1000bp)

99%

rRNA tRNA pRNA mtRNA ncRNA

Need

Whole-Transcript-amplification

over 10,000-fold amplification

Detectors

>100ng Total RNA

(>10,000 cell)

Microarray

mRNA-seq

Single-Cell RNA-Seq

A novel single-cell RNA-seq method

Single-cell whole-transcript-amplification methods

Poly-A tailing (Target: all cDNA)

Reproducibility and sensitivity

Brady et al. 1990 → Microarray: Kurimoto et al. 2006 → Seq (Solid): Tang et al. 2009 → Microarray / Seq (Illumina): Quartz-Seq/Chip

Template switching (Target: full length cDNA)

SMARTer system

- Seq (RPKM, Illumina): Smart-Seq 2012 — *Transcript Coverage*
- Seq (tag count, Illumina): STRT-Seq 2011 — *High-throughput (5' end)*

In vitro transcription-based

- Seq (tag count, Illumina): Cel-Seq 2012 — *High-throughput (3' end)*

Quartz-Seq

A novel single-cell RNA-seq method

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Genome Biology 2013, **14**:R31 (17 April 2013)
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Daehwan Kim, Geo Pertea, Cole Trapnell, Harold Pimentel, Ryan Kelley, Steven L Salzb
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3. **Research** Open Access Highly accessed
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John Abramyan, Daleen Badenhorst, Kyle K Biggar, Glen M Borchert, Christopher W Bot
Edward L Braun, Anne M Bronikowski, Benoit G Bruneau, Leslie T Buck, Blanche Capel,
Czerwinski, Kim D Delehaunty, Scott V Edwards, Catrina C Fronick, Matthew K Fujita, Lu
Richard E Green, Wilfried Haerty, Ramkumar Hariharan, LaDeana H Hillier, Alisha K Holl
Janzen, Cyriac Kandoth, Lesheng Kong, Jason de Koning, Yang Li *et al.*
Genome Biology 2013, **14**:R28 (28 March 2013)
Abstract | Provisional PDF | PubMed | ▸Editor's summary

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Naomi Attar

Editor's Picks

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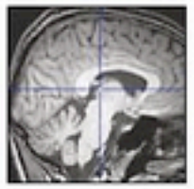
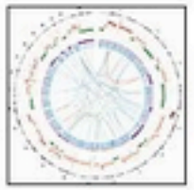
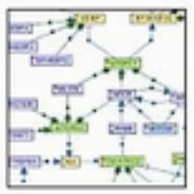


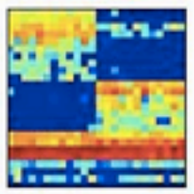
Neurogenomics
From GWAS to Neanderthal genomes: Fisher and Deriziotis review what genomics can teach us about speech, language and their associated disorders
Genome Biology 2013, **14**:204



The HeLa genome
David Mittelman and John Wilson steer clear of the controversy, and offer a scientific perspective on the HeLa cell genome
Genome Biology 2013, **14**:111



p53 mTOR stress
p53 activation results in mTOR inhibition and global repression of protein translation in response to oncogenic and energy stress
Genome Biology 2013, **14**:R32



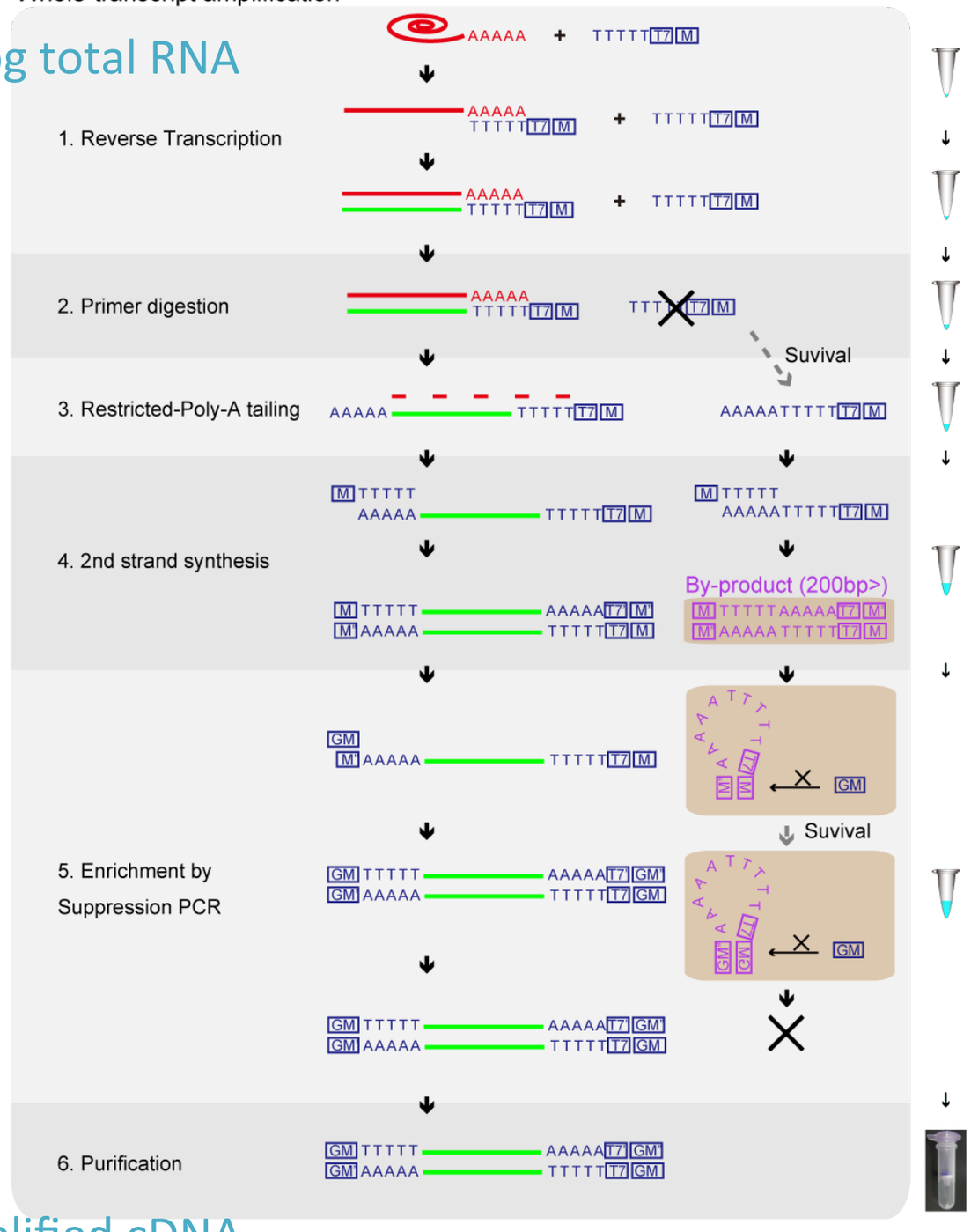
Quartz-Seq
Quartz-Seq is a method for pe rming single cell RNA-seq which displays high reproducibility
Genome Biology 2013, **14**:R31

Quartz-Seq

A novel single-cell RNA-seq method

10 pg total RNA

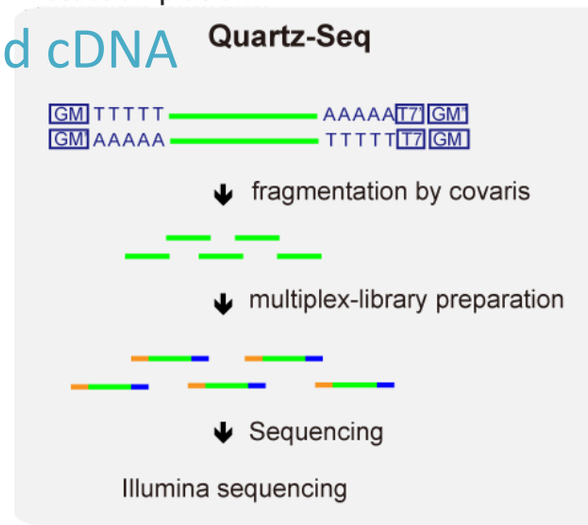
Whole-transcript amplification



Amplified cDNA

Amplified cDNA

Detection platform



Sequence Library DNA

Quartz-Seqの改善点

A novel single-cell RNA-seq method

適切な酵素の選択

MighthyAmp Ver.2 (TaKaRa Bio)

副産物の増幅を抑える = 190,000 倍量のPrimer

Suppression PCR, Exonuclease I Digestion, Reaction time of Poly-A tailing

mRNAからcDNAを効率良く作る

Optimal annealing temperature etc.

Quartz-Seqの性能

A novel single-cell RNA-seq method

8割の遺伝子発現を検出

これまでは3.5割程度

高い再現性を達成 (相関係数0.93)

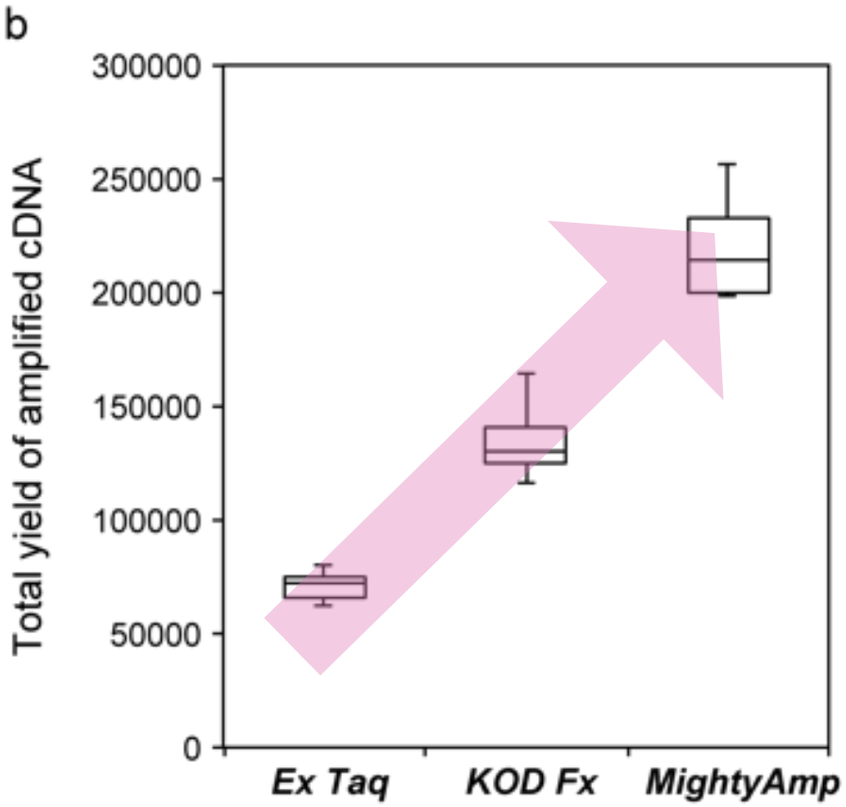
これまでの最高は0.7

同じ培養条件/細胞周期の細胞ゆらぎの測定に始めて成功

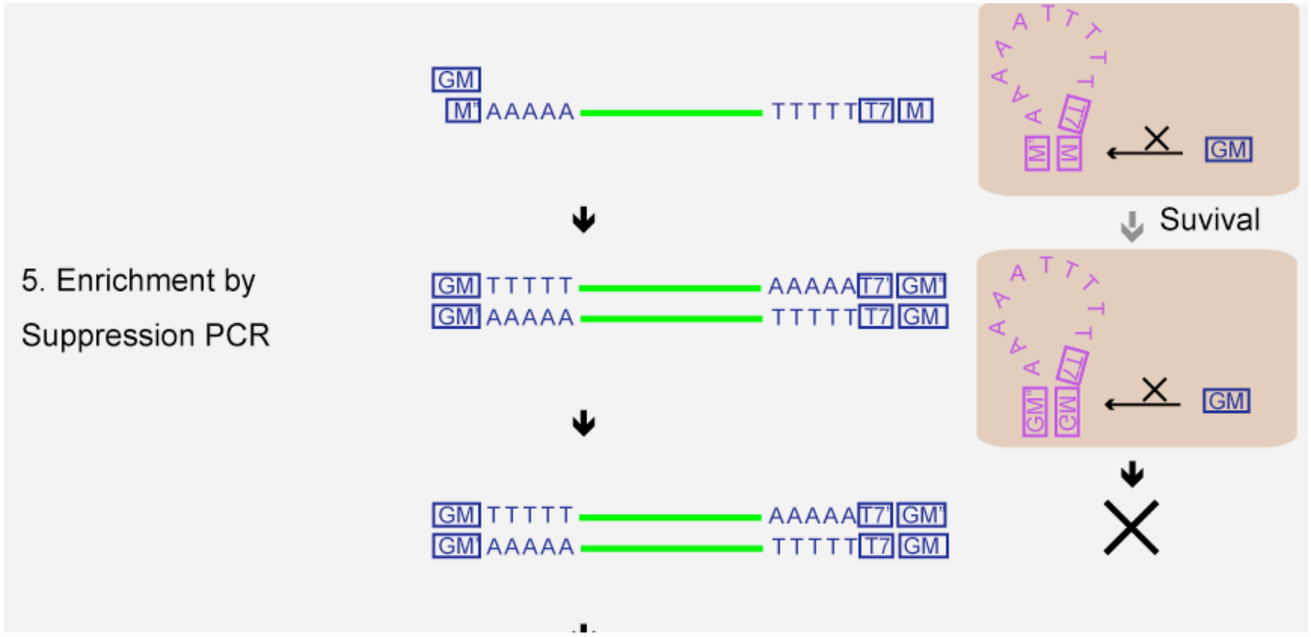
これまでは細胞種類の分類すら困難

Quartz-Seqの改善点

DNAポリメラーゼの最適化



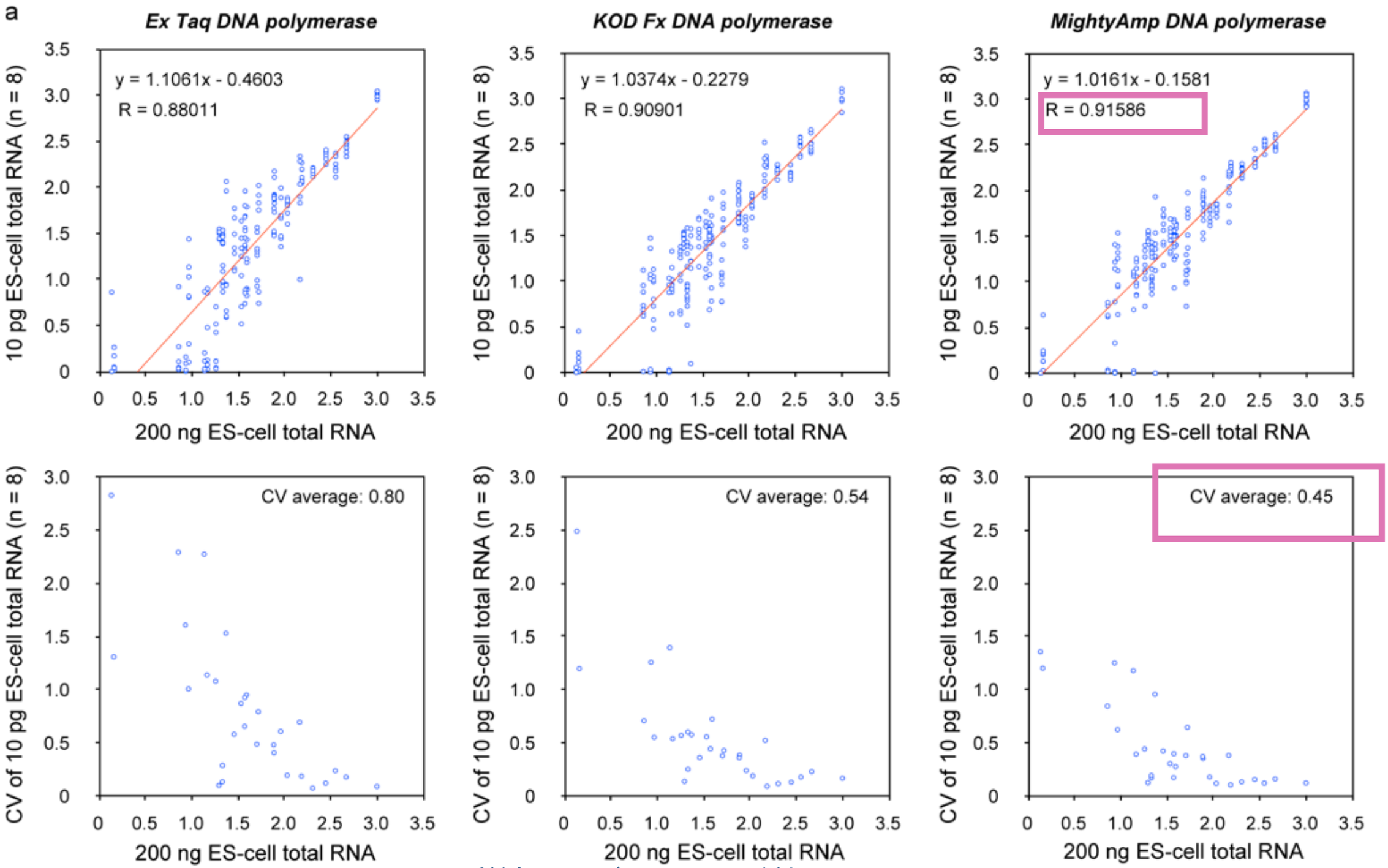
既存法　　Quartz



cDNAの収量が約5倍に

Quartz-Seqの改善点

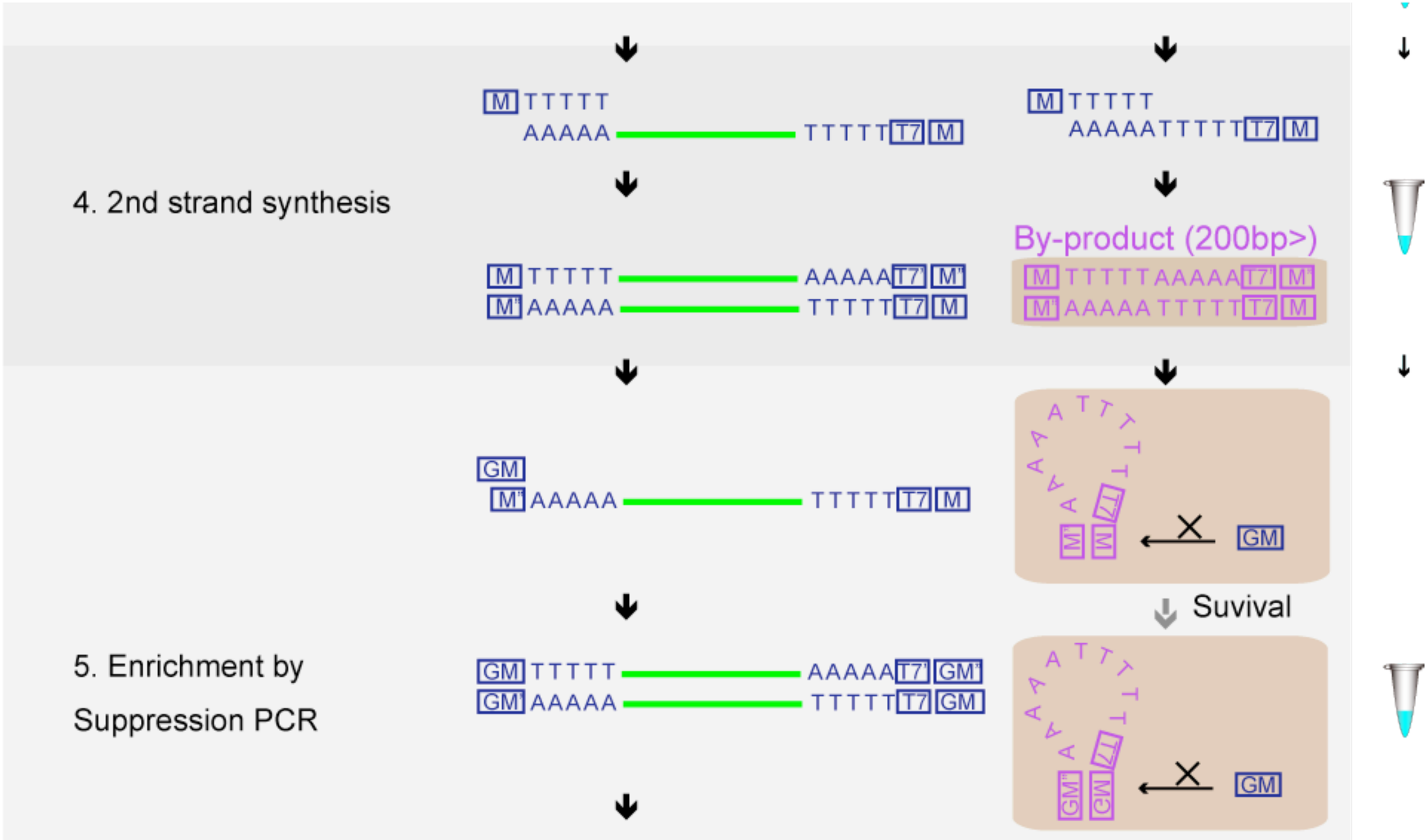
DNAポリメラーゼの最適化



増幅のばらつきが約1/2に

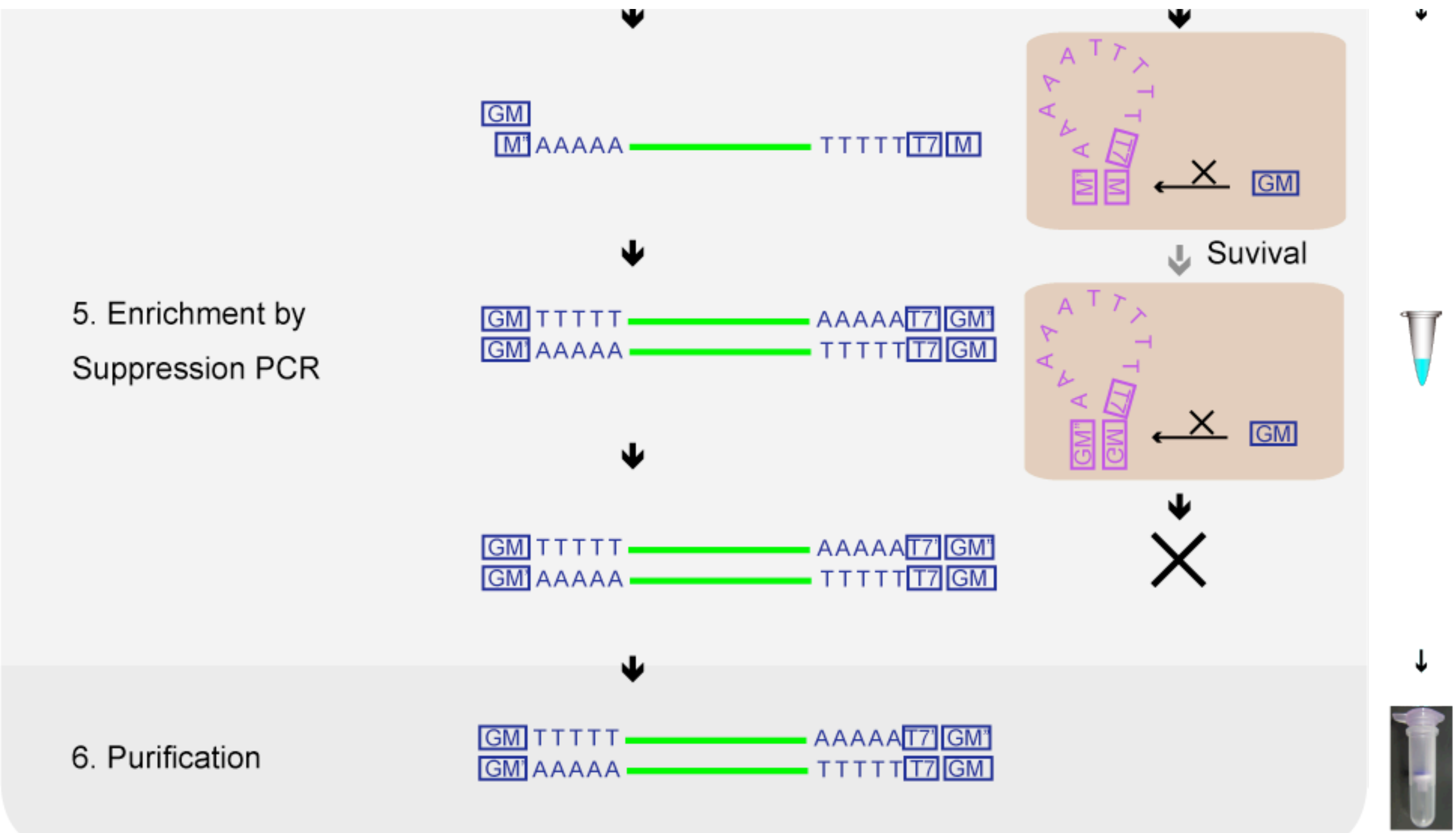
副産物の増幅を抑える = 190,000 倍量のPrimer

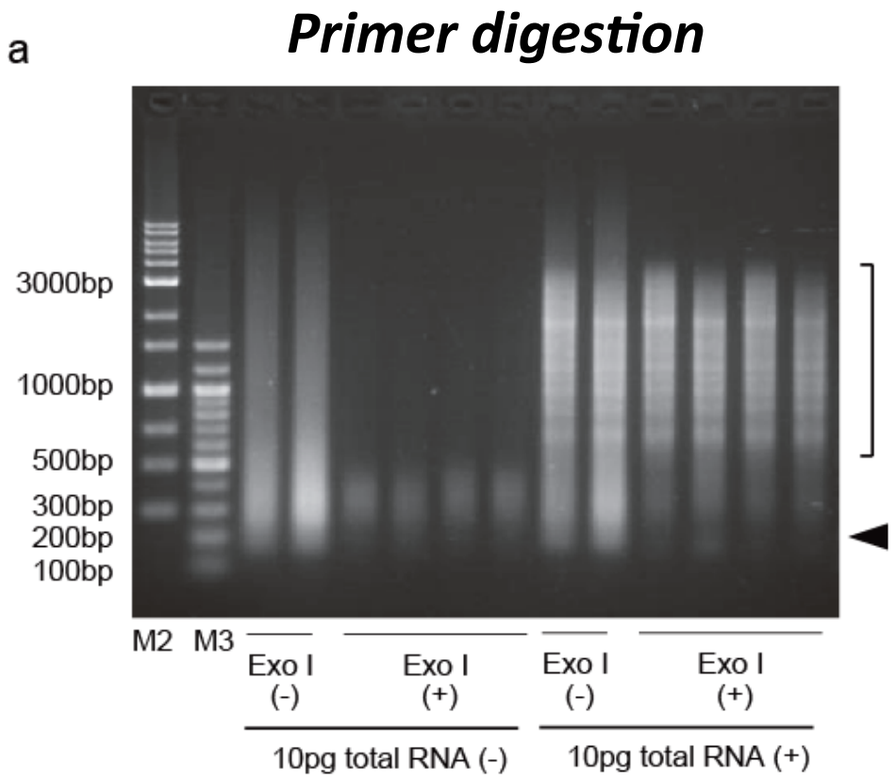
Suppression PCR

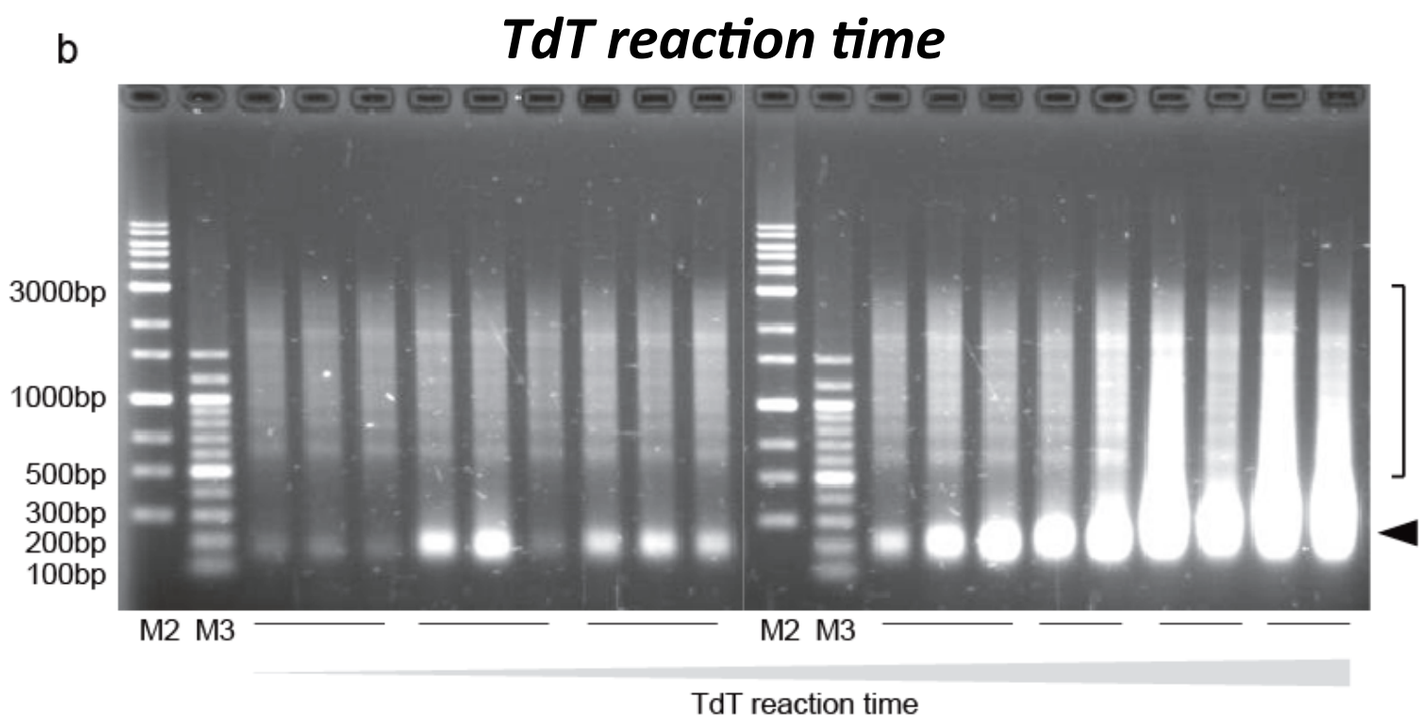


副産物の増幅を抑える = 190,000 倍量のPrimer

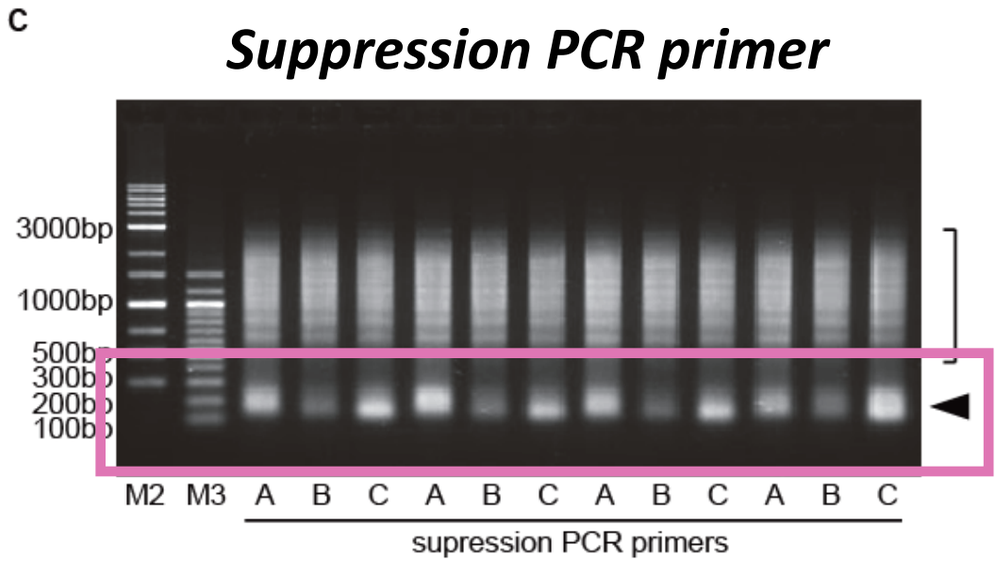
Suppression PCR

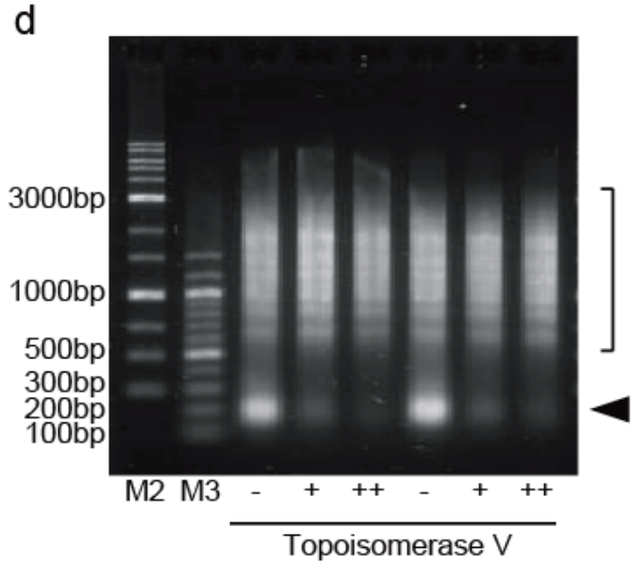


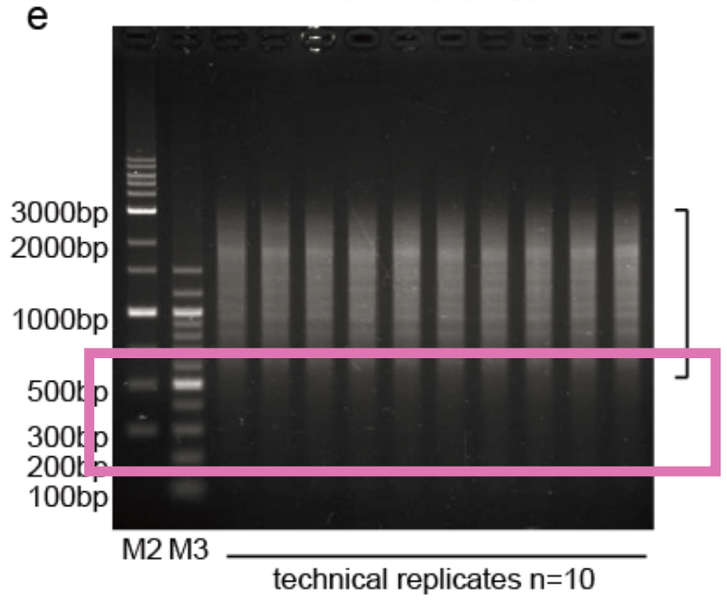




Combination





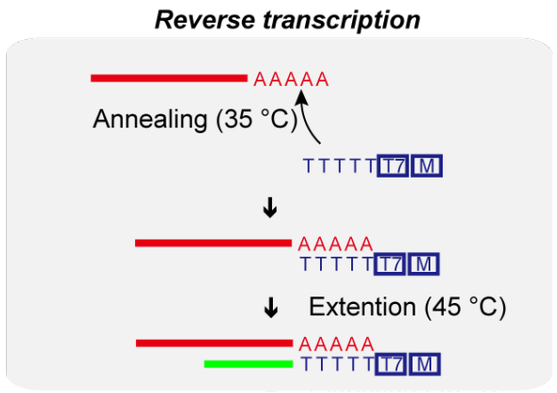


副産物の増幅を抑える = 190,000 倍量のPrimer

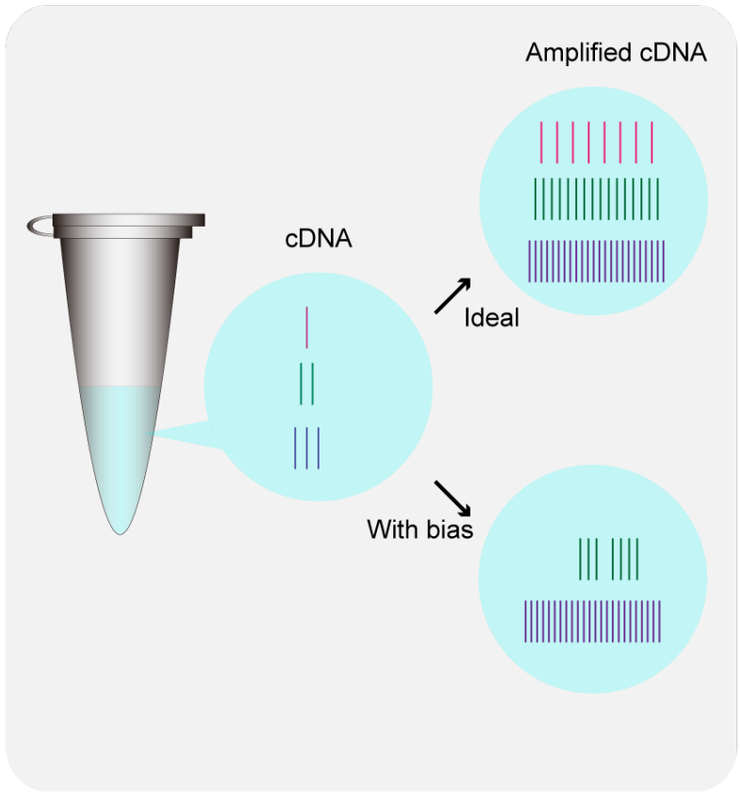
Suppression PCR

副産物を複雑な手技なし(ゲル切り出し)に、ほぼ完全に消去!

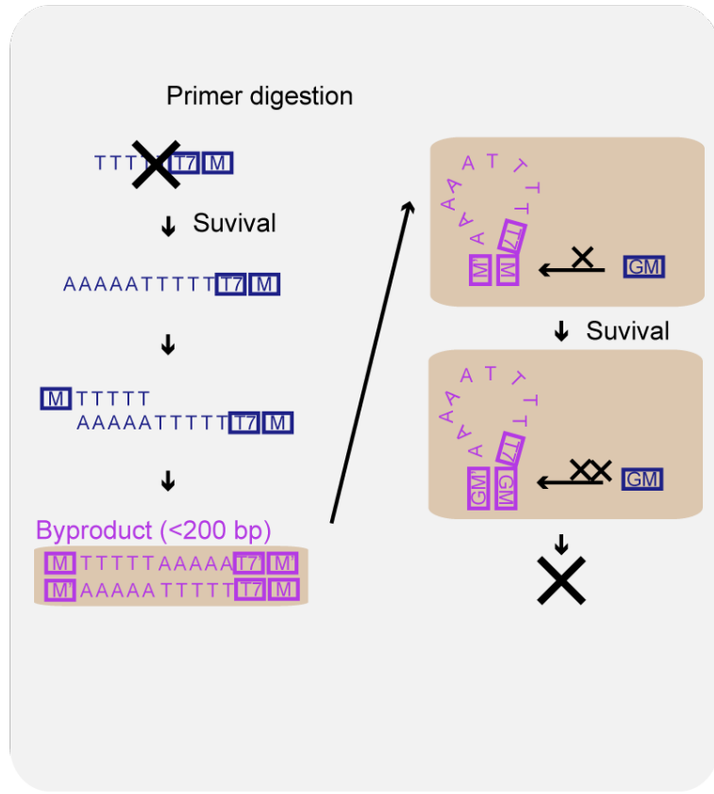
Capture variability

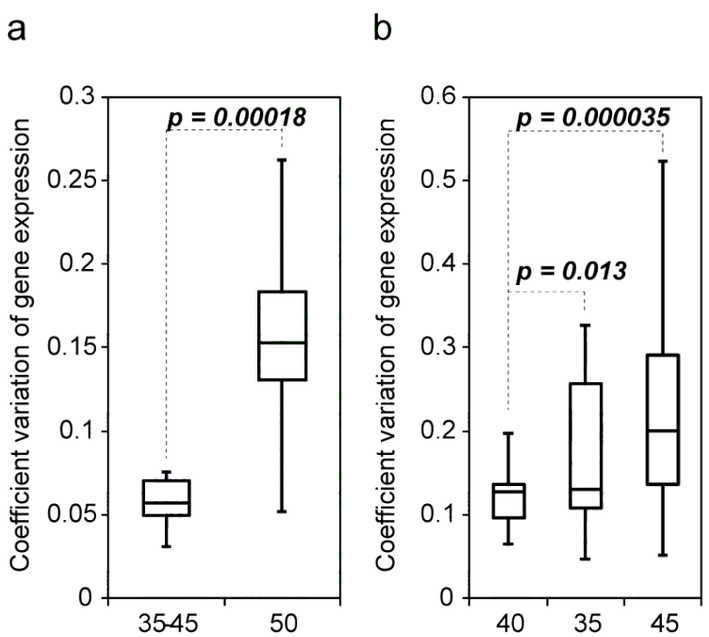


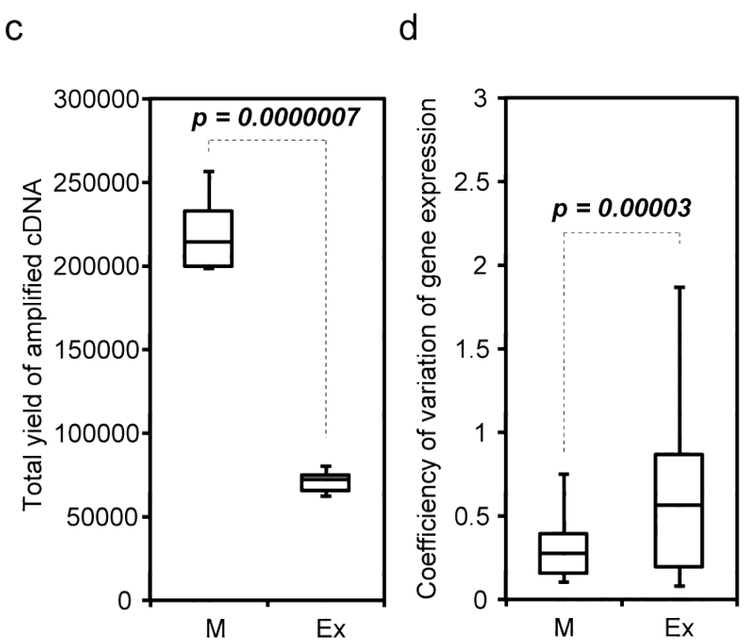
Sensitivity and bias in PCR

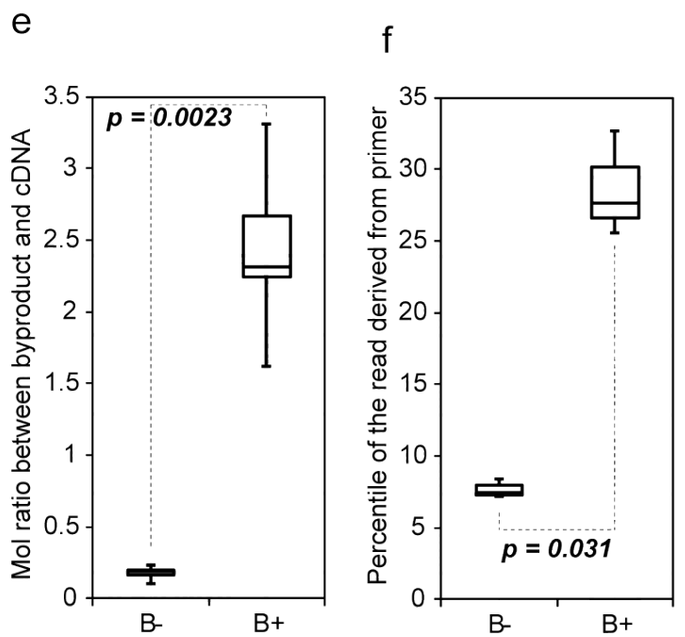


Byproducts









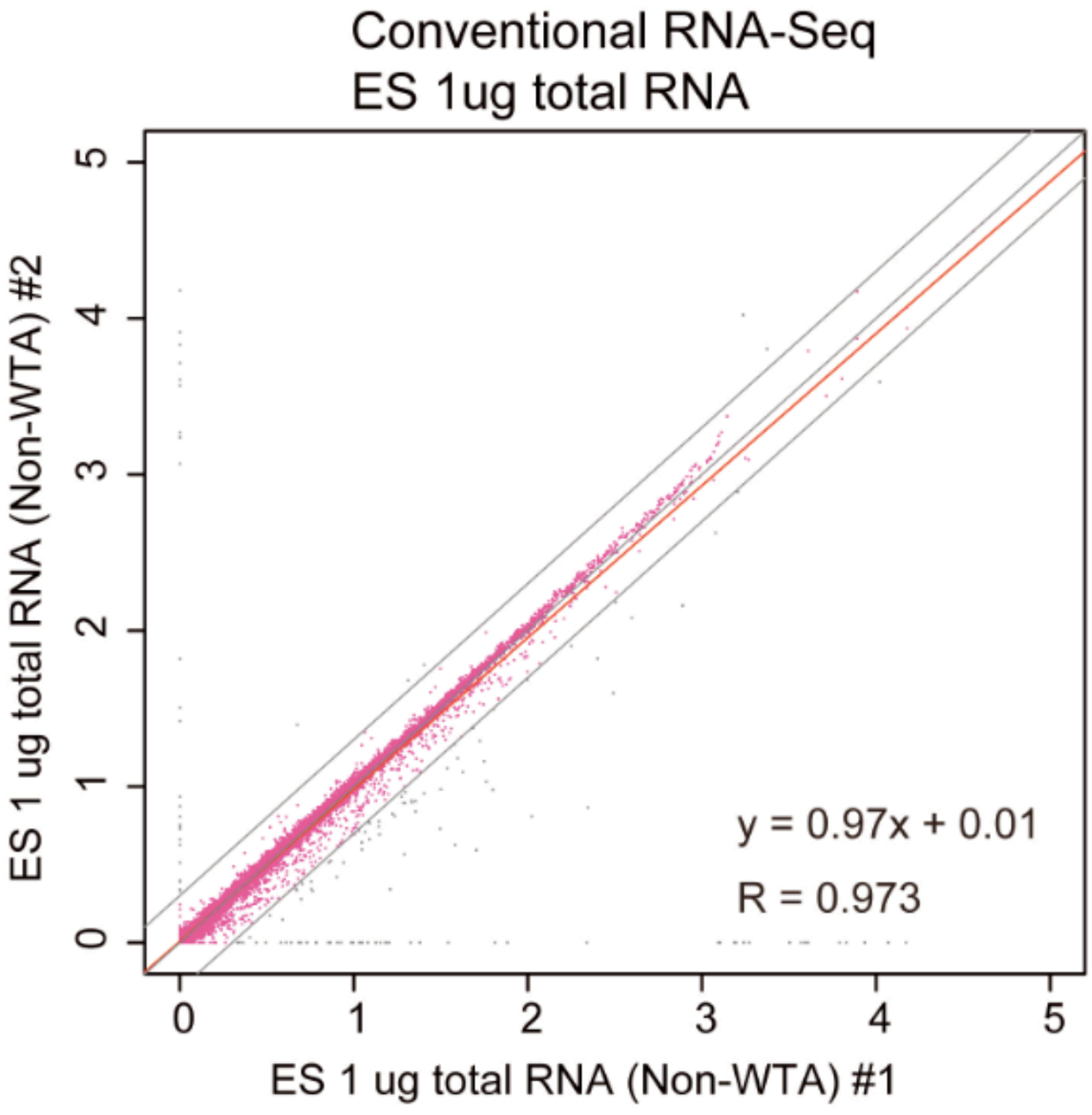
RNA捕捉ばらつきが1/3へ

cDNA収量が約5倍に
cDNA収量ばらつきが1/2へ

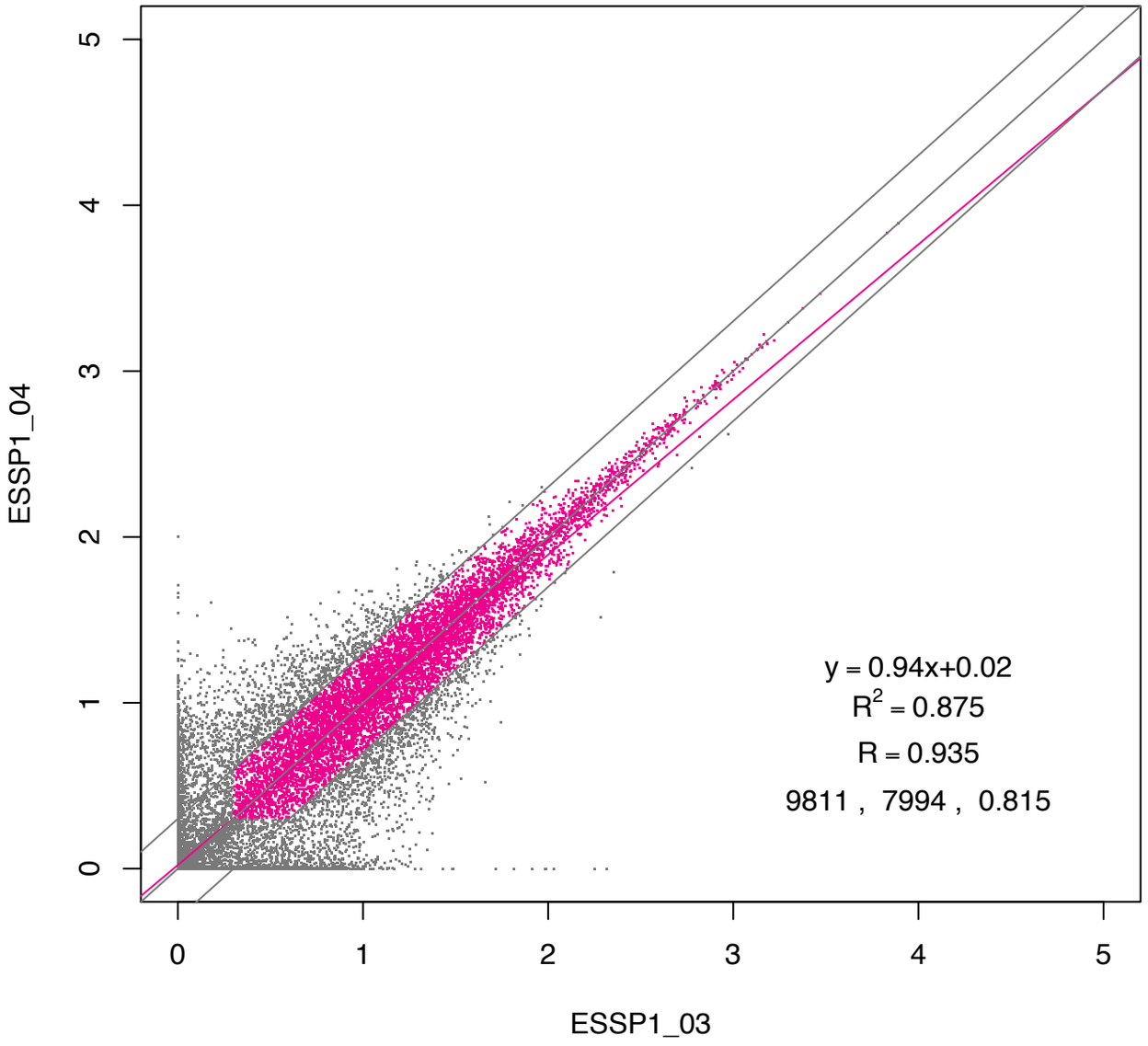
副産物をほぼ完全に消去

Quartz-Seq

A novel single-cell RNA-seq method



Quartz-Seq (Technical replicates)

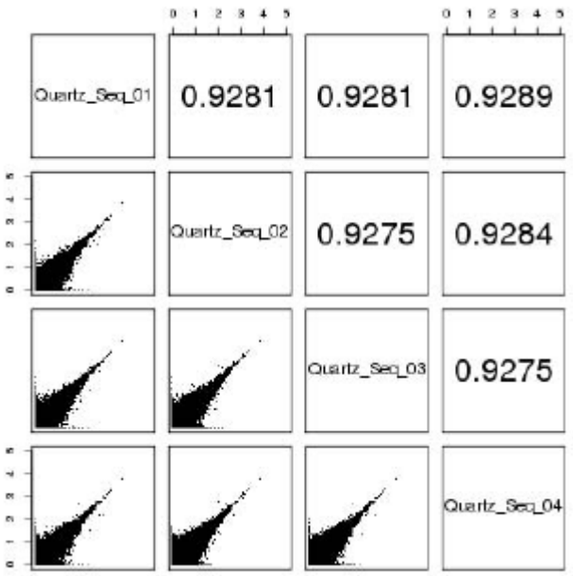


(Sasgawa Y. and Nikaido I. et. al. 2013)

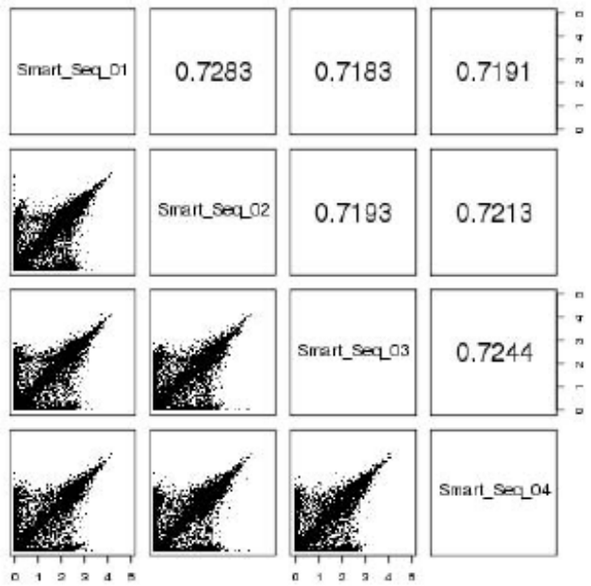
Single-Cell RNA-seq

10 pg Total RNA (0.1 pg mRNA) からの RNA-seq

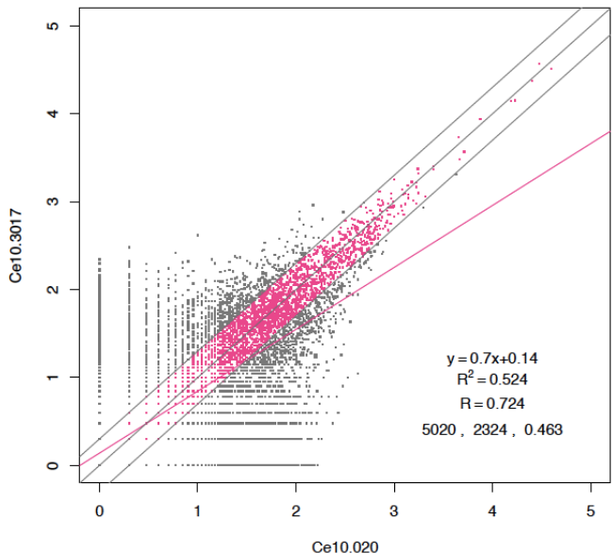
Quartz-Seq



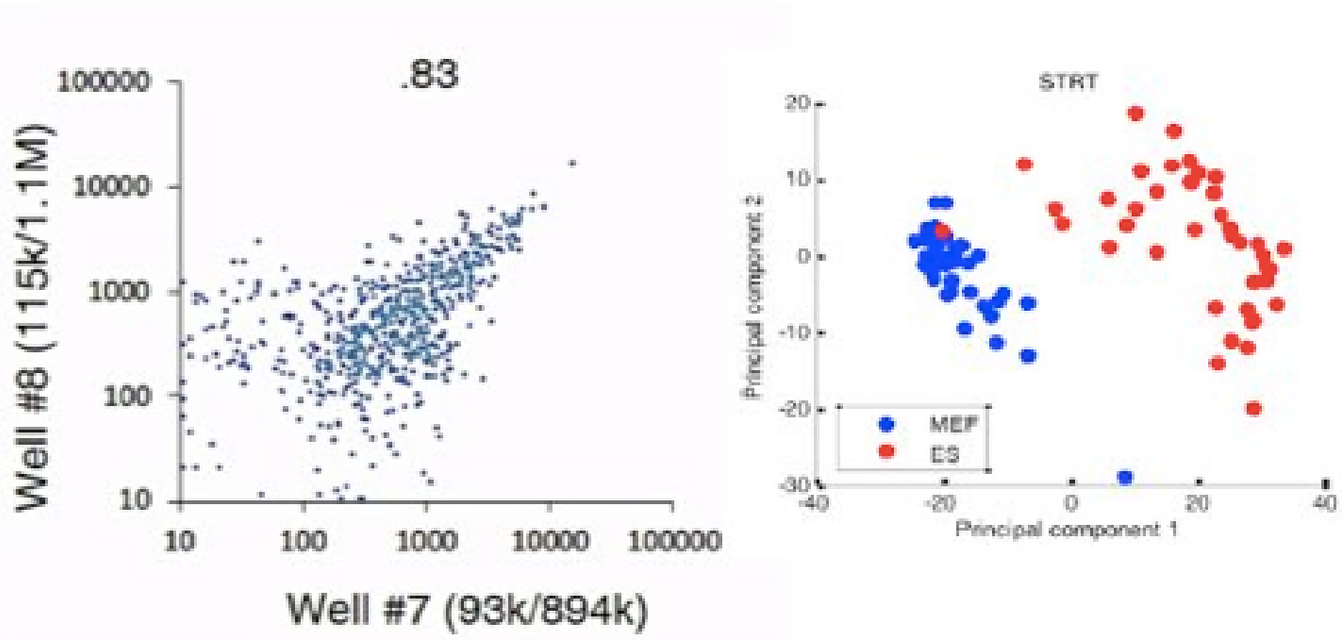
Clontech UL kit, Smart-Seq
Ramskold D. et al., 2012



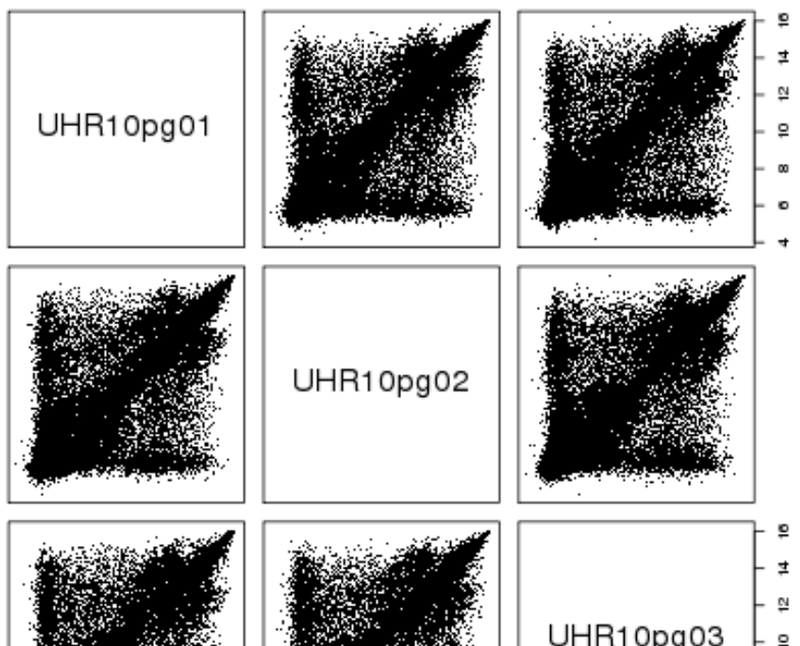
Hashimshony et al., 2012 (CEL-Seq)



Islam et al., 2011

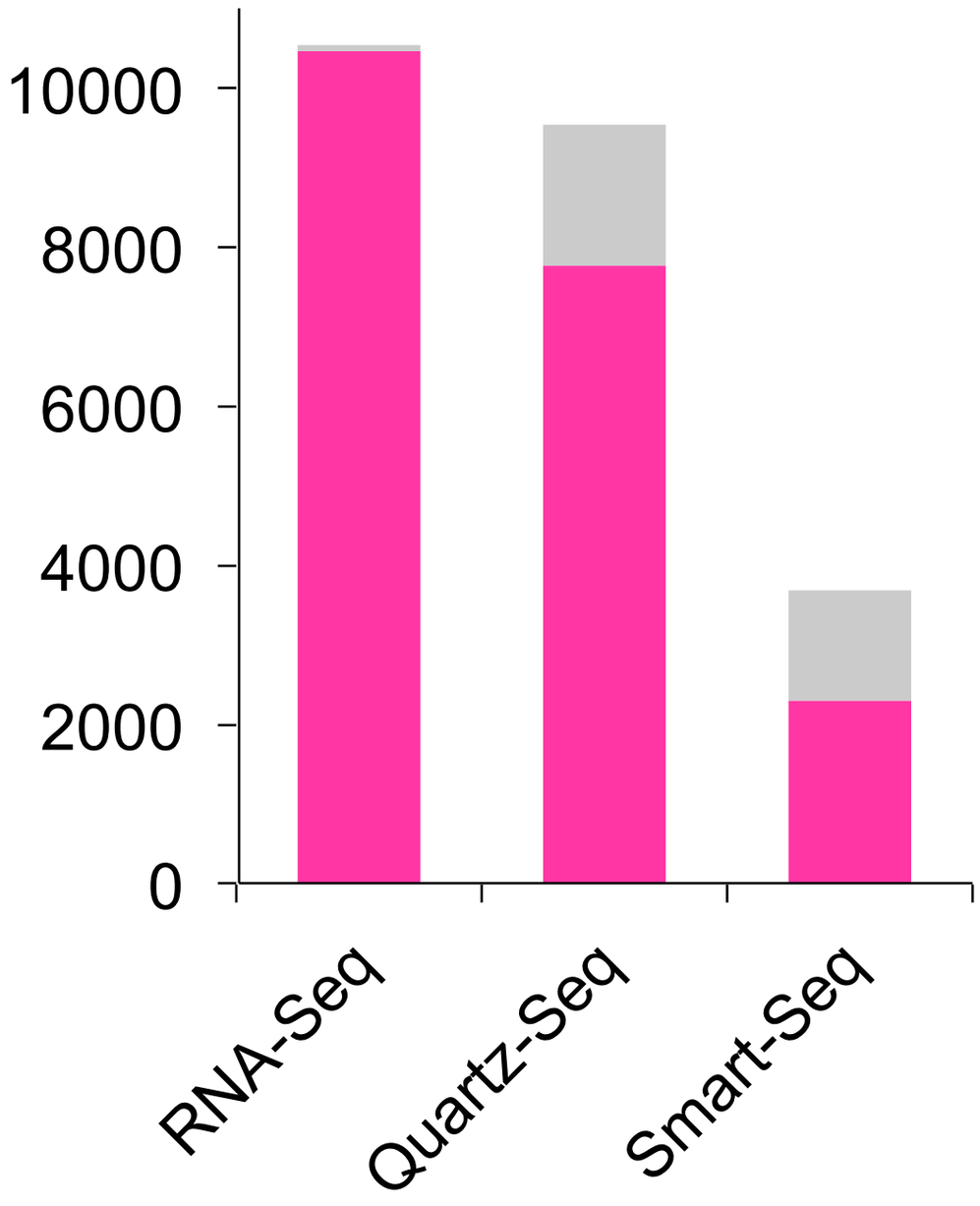


Fan JB et al., 2012



検出遺伝子数

Quartz-Seq vs. Smart-Seq



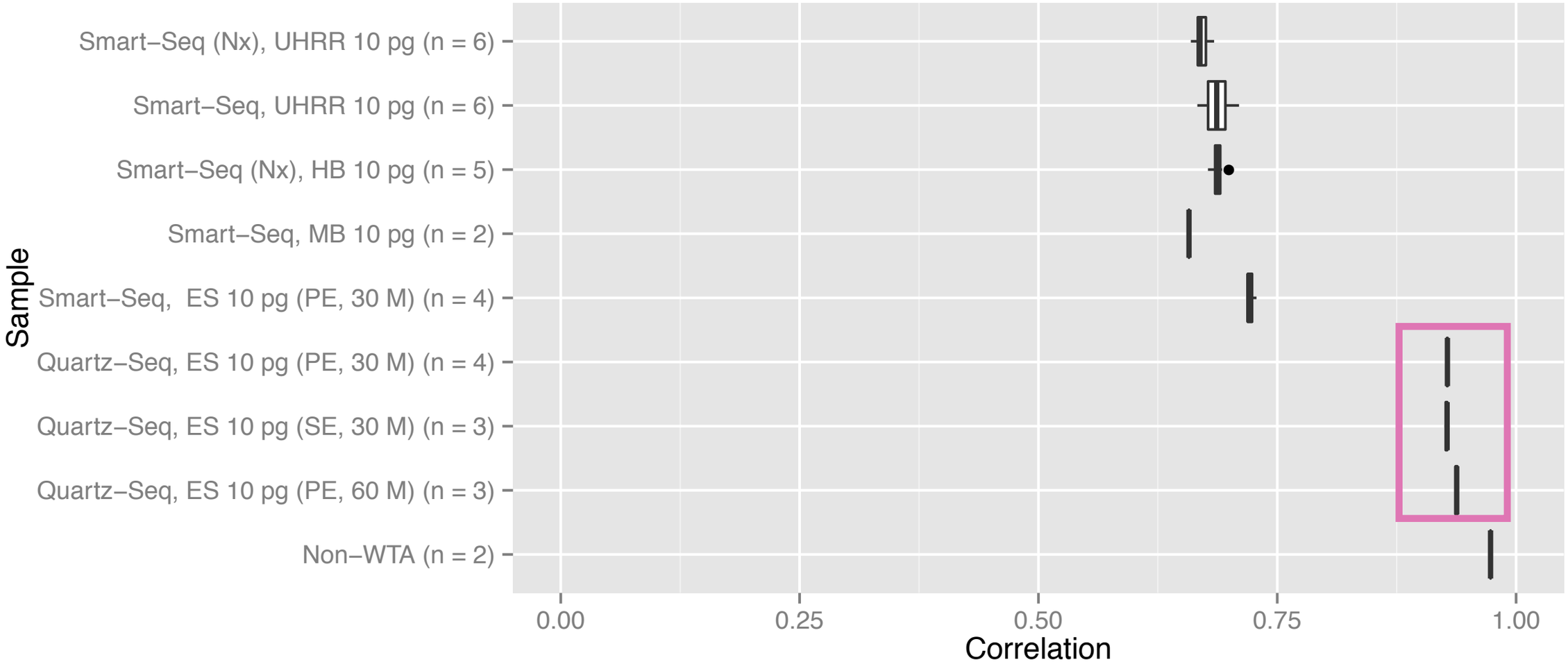
検出できる遺伝子

Quartz-Seq: 8,000

Smart-Seq: 3,000

Sensitivity

独立した1細胞RNA-Seqの相関係数



Smart-Seqより圧倒的に再現性が高いQuartz-Seq

(0.70から0.93へ)

Primer contaminations

Quartz-Seq vs. Smart-Seq

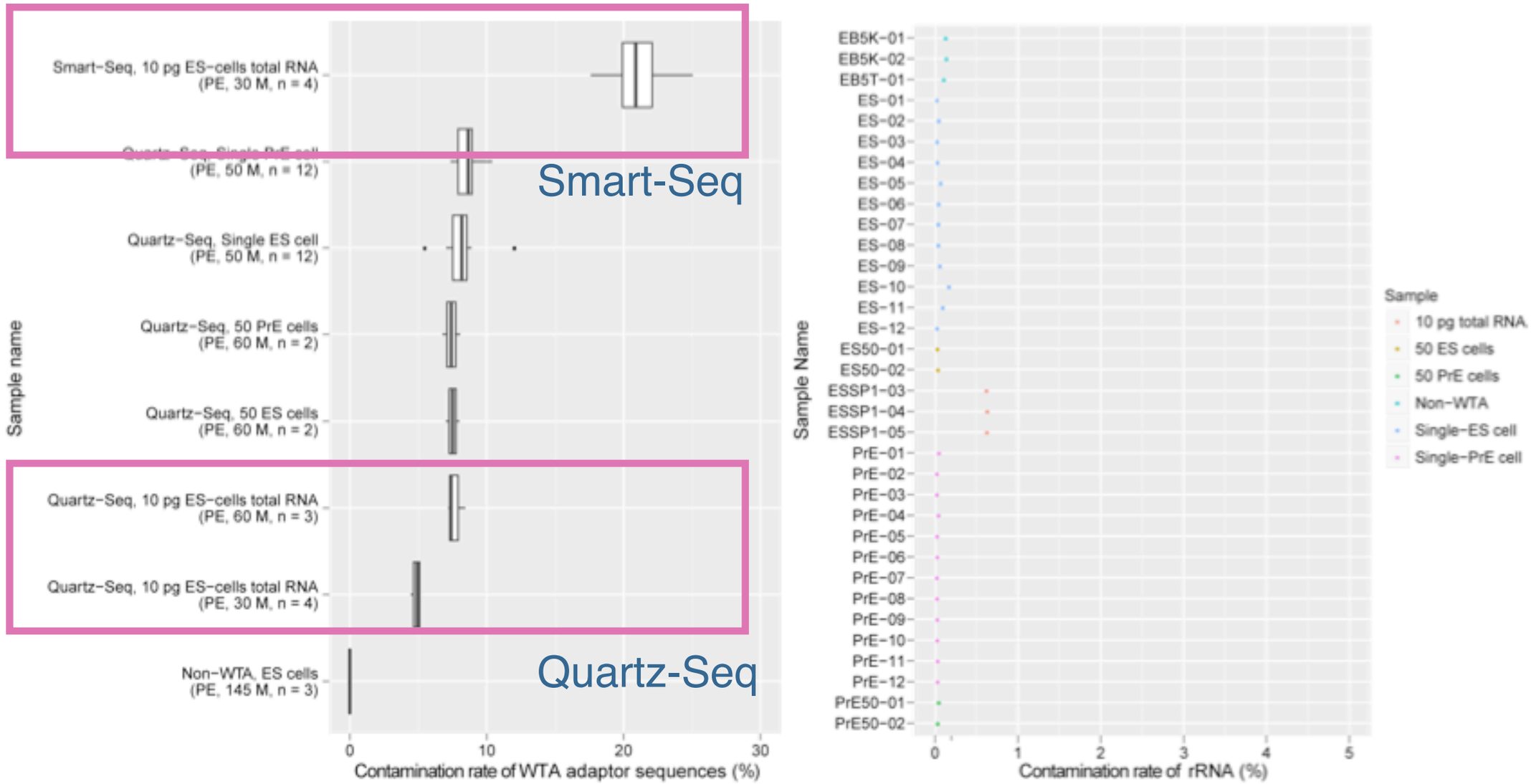


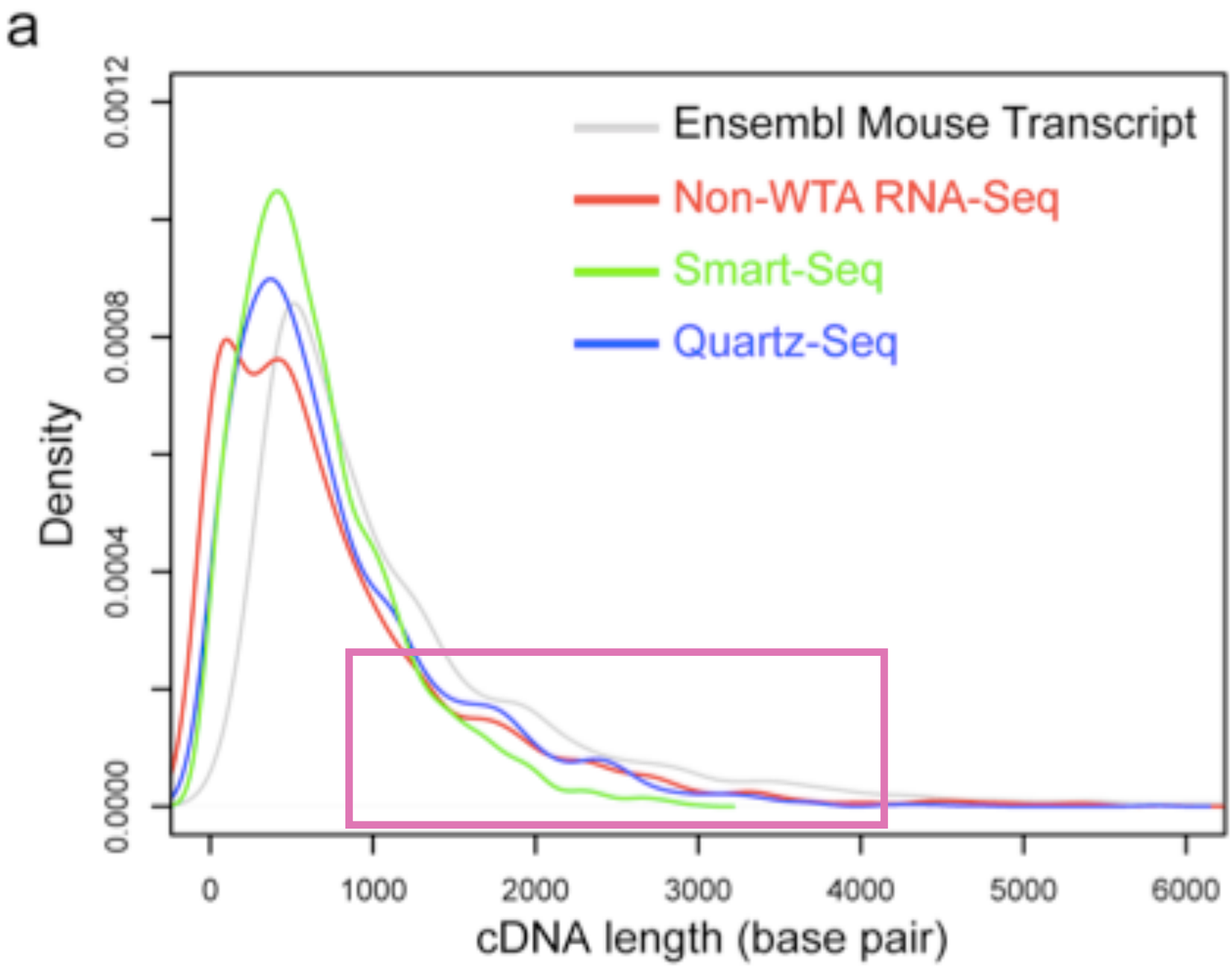
Figure S8 Percentage of sequence reads of the suppression PCR primer or rRNA.
The left panel shows the contamination rate of the sequence read from the WTA adaptor sequences. The X-axis indicates the contamination percentage of all sequence reads. The right panel shows the contamination rate of rRNA. The X-axis indicates the percentage of the rRNA contamination in all sequence reads.

Smart-Seqは20%は無駄なシーケンス = シーケンスコストが2割増し

Quartz-Seqはわずか8%

cDNA Length

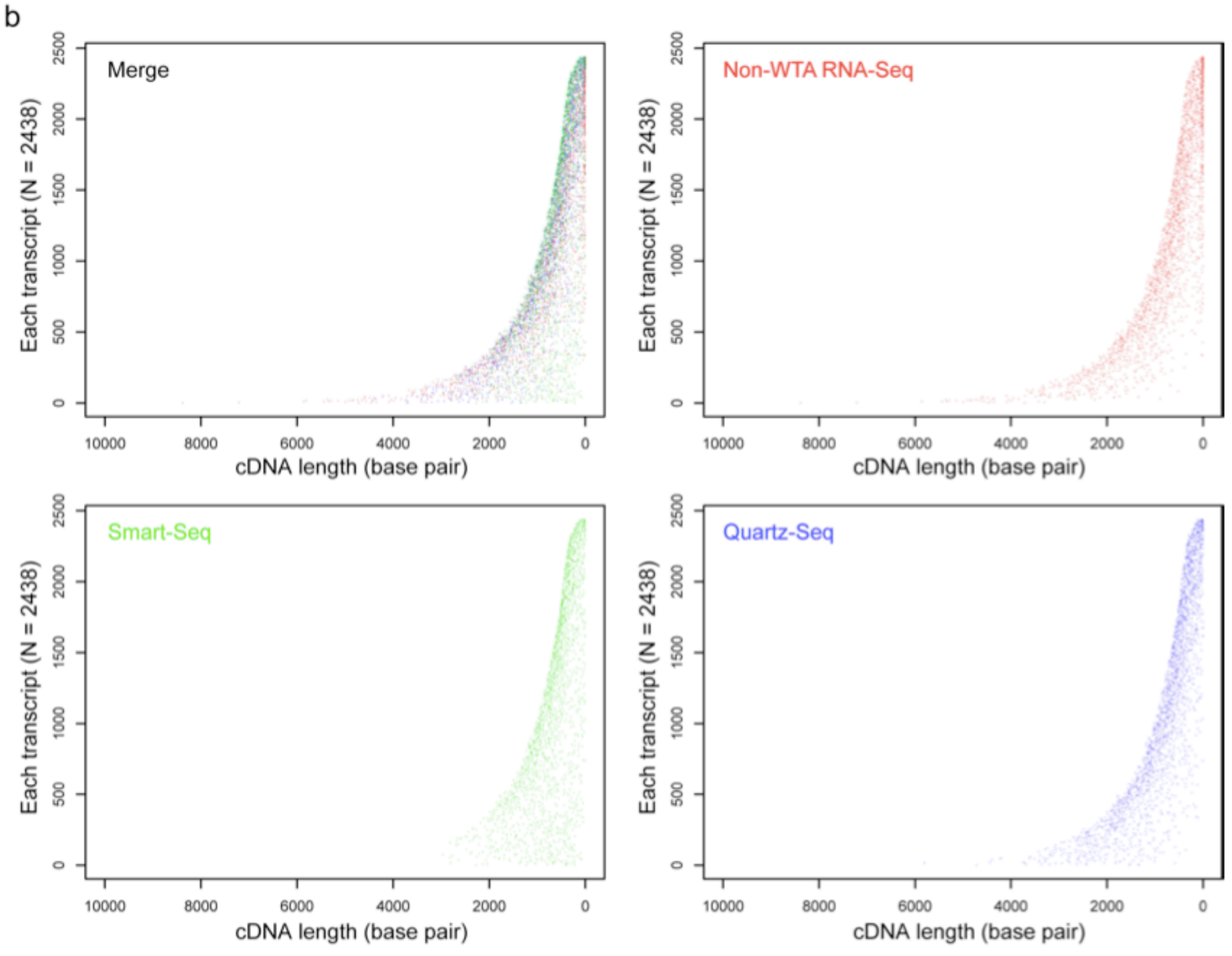
Quartz-Seq vs. Smart-Seq



Smart-SeqよりQuartz-Seqのほうが長い転写産物を捉えられる

cDNA Length

Quartz-Seq vs. Smart-Seq



50 cellsでも利用できる

Quartz-Seq

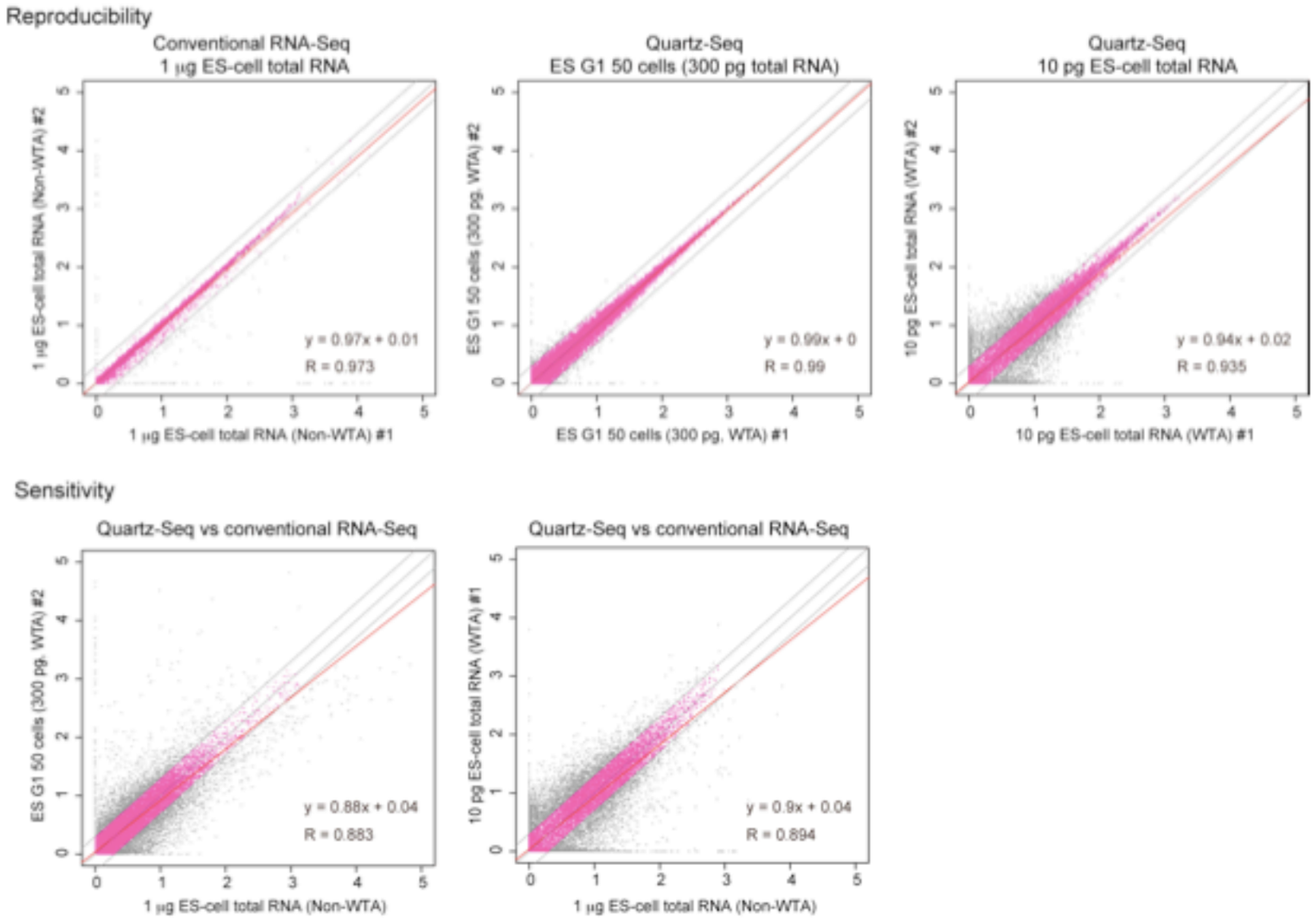


Figure S17 Scatter plots of conventional RNA-Seq and Quartz-Seq using 50 ES cells at the G1 phase of the cell cycle and Quartz-Seq using 10 pg of total ES RNA.
The upper panel shows the reproducibility of technical replicates of conventional RNA-Seq, Quartz-Seq (50 cells) and Quartz-Seq (10 pg of total ES RNA). The lower panel shows the sensitivity of Quartz-Seq with 50 single cells and 10 pg of total ES RNA. The left scatterplot is same in Figure 2. The respective Pearson correlation (R) and regression equation are shown in each plot. The gray lines indicate a two-fold change and y = x. The red line is a linear regression. The red points indicate a transcript with an FPKM expression larger than 1.0 and exhibited less than two-fold expression changes between technical duplicates.

Cost

Quartz vs. Smart

Quartz

500円/amplification

SMARTer (CloneTech)

>10,000円/amplification

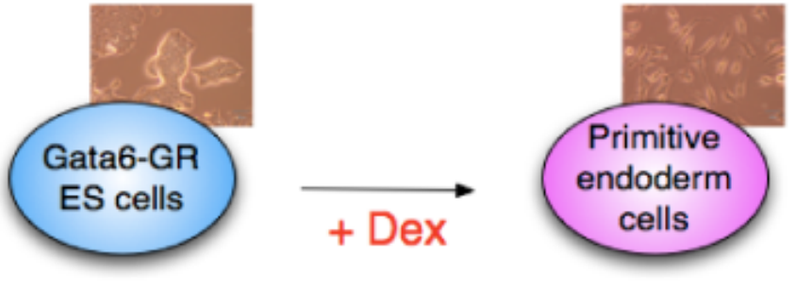
1. 異なる細胞の種類を見分ける

cellular heterogeneity between different cell-types

2. 同一細胞集団の発現ゆらぎを区別する

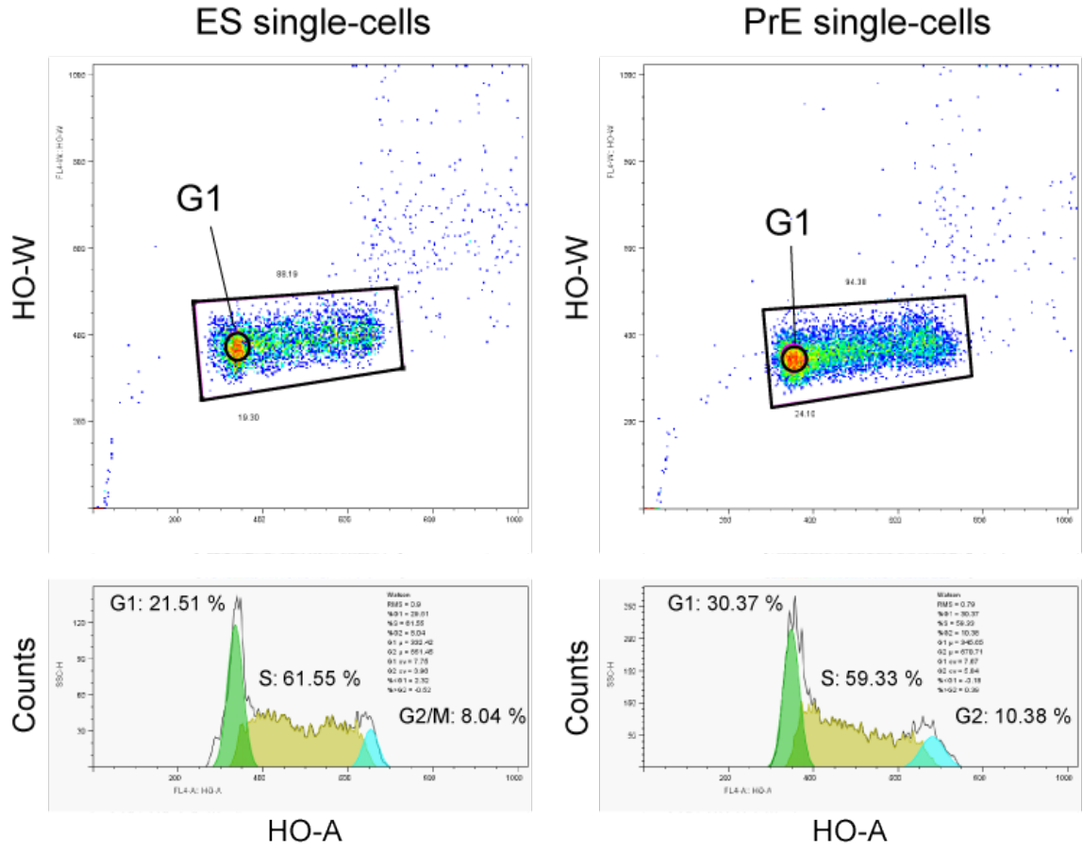
cellular heterogeneity in same culture condition

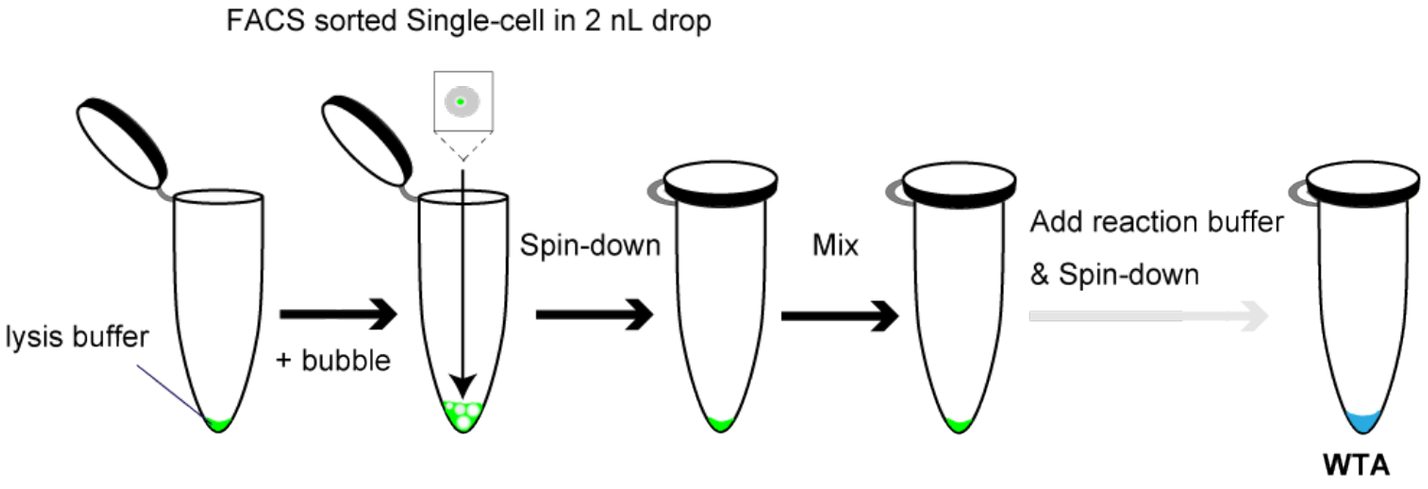
1. Difference of differentiated state

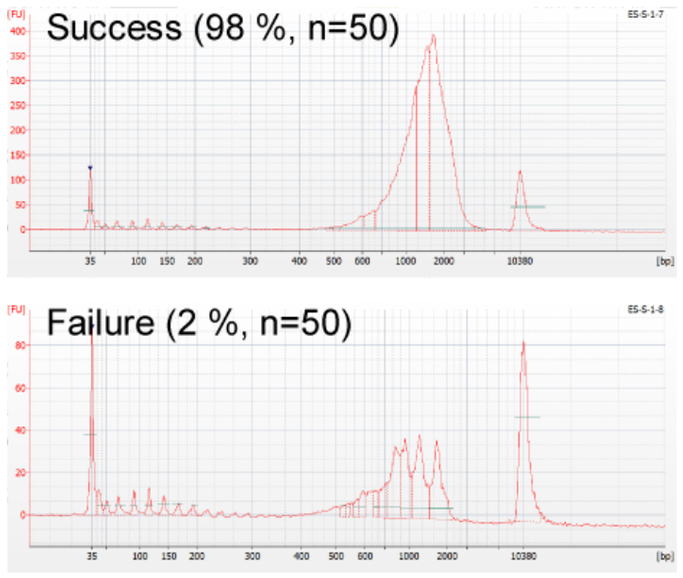


1. Hormone-induced transcriptional activation
(Activation in 15 min; repression in 30 min)
2. Uniformity of differentiation(100%)

(Niwa, BMC Dev. Biol. 2007)



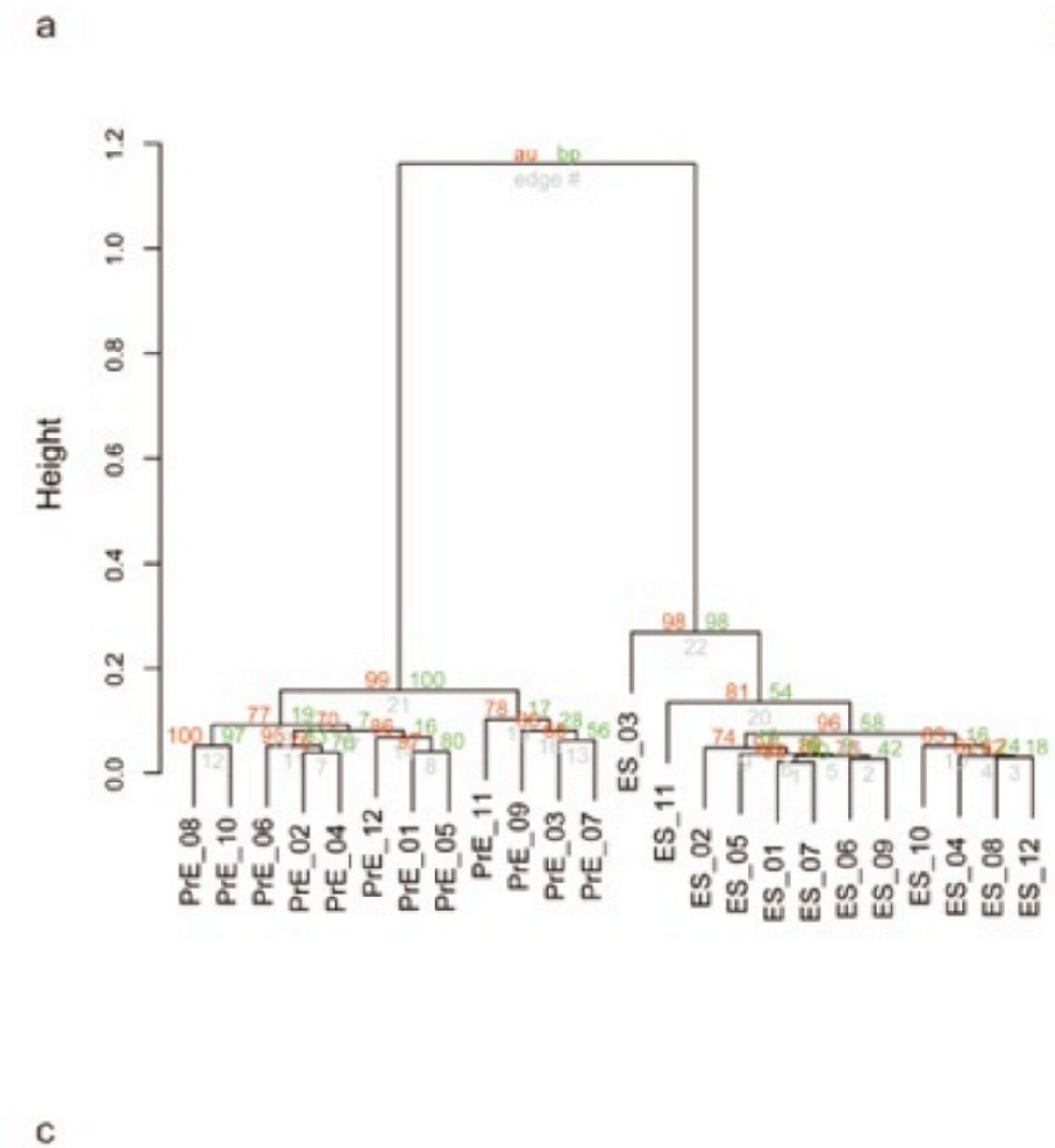


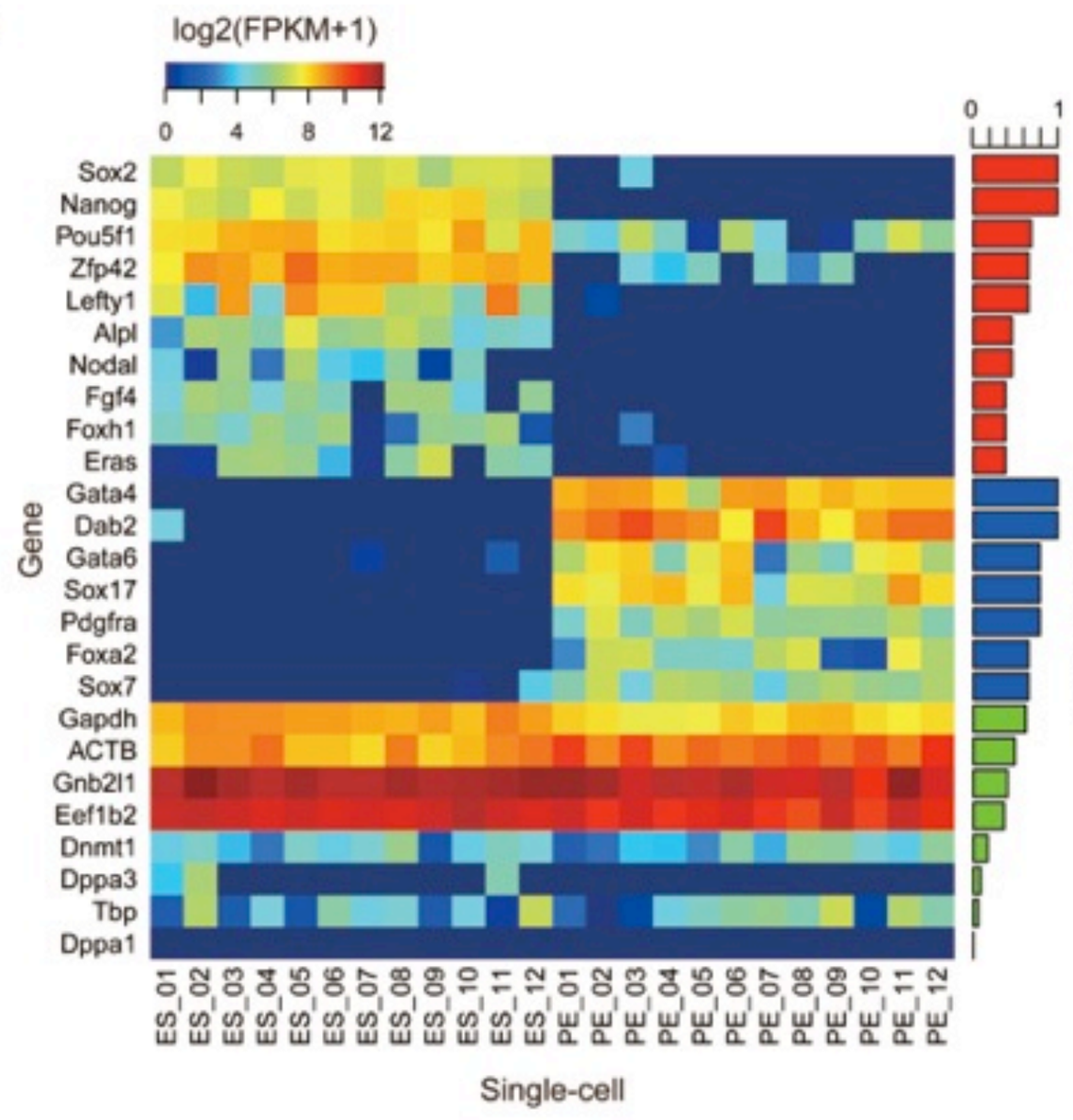


1. 異なる細胞の種類を見分ける

cellular heterogeneity between different cell-types

single-cell sorting → (12 ES cells / 12 PrE cells) → single-cell RNA-seq





c

1. 異なる細胞の種類を見分ける

cellular heterogeneity between different cell-types

2. 同一細胞集団の発現ゆらぎを区別する

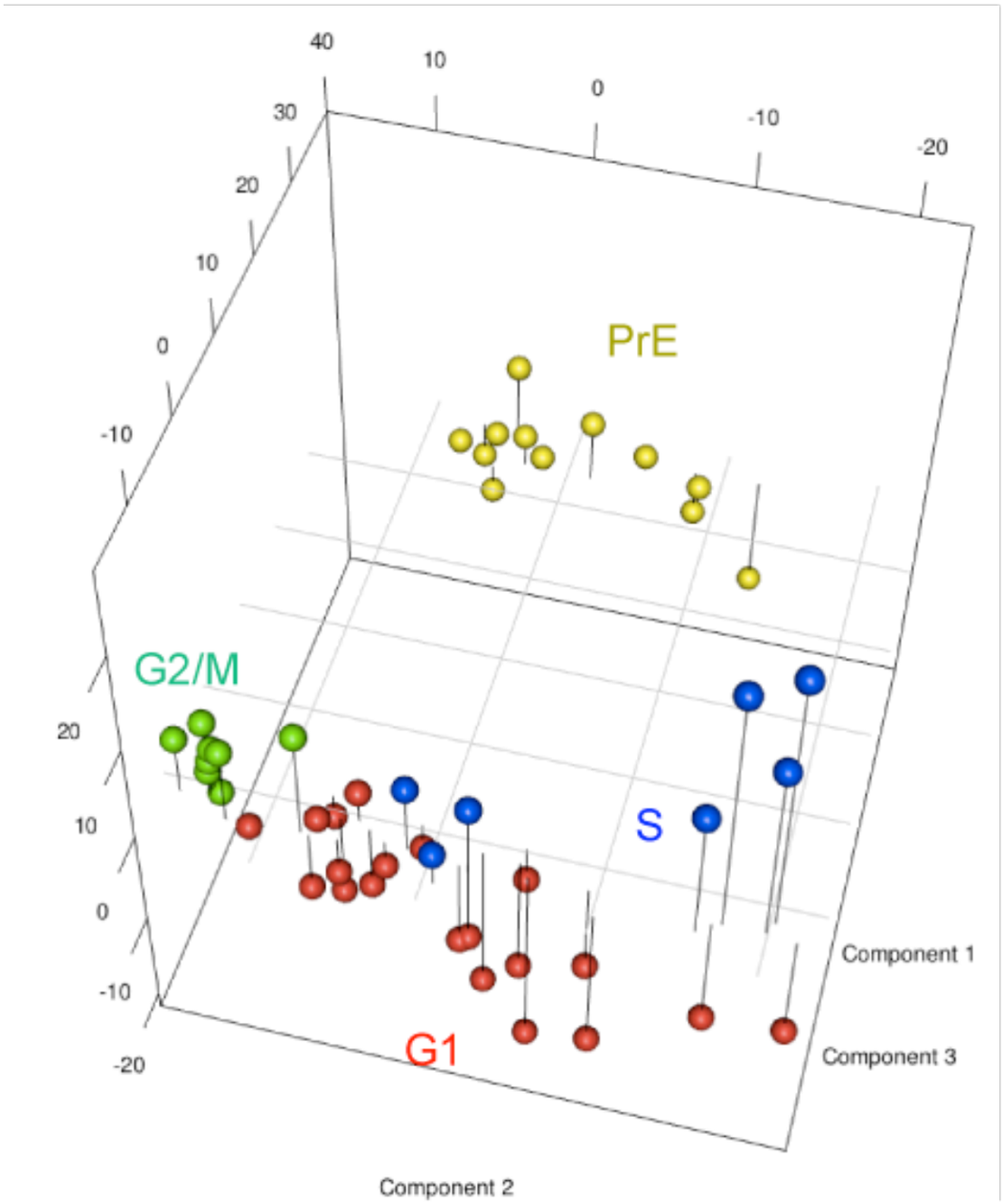
cellular heterogeneity in same culture condition

2. 同一細胞集団の発現ゆらぎを区別する (細胞周期)

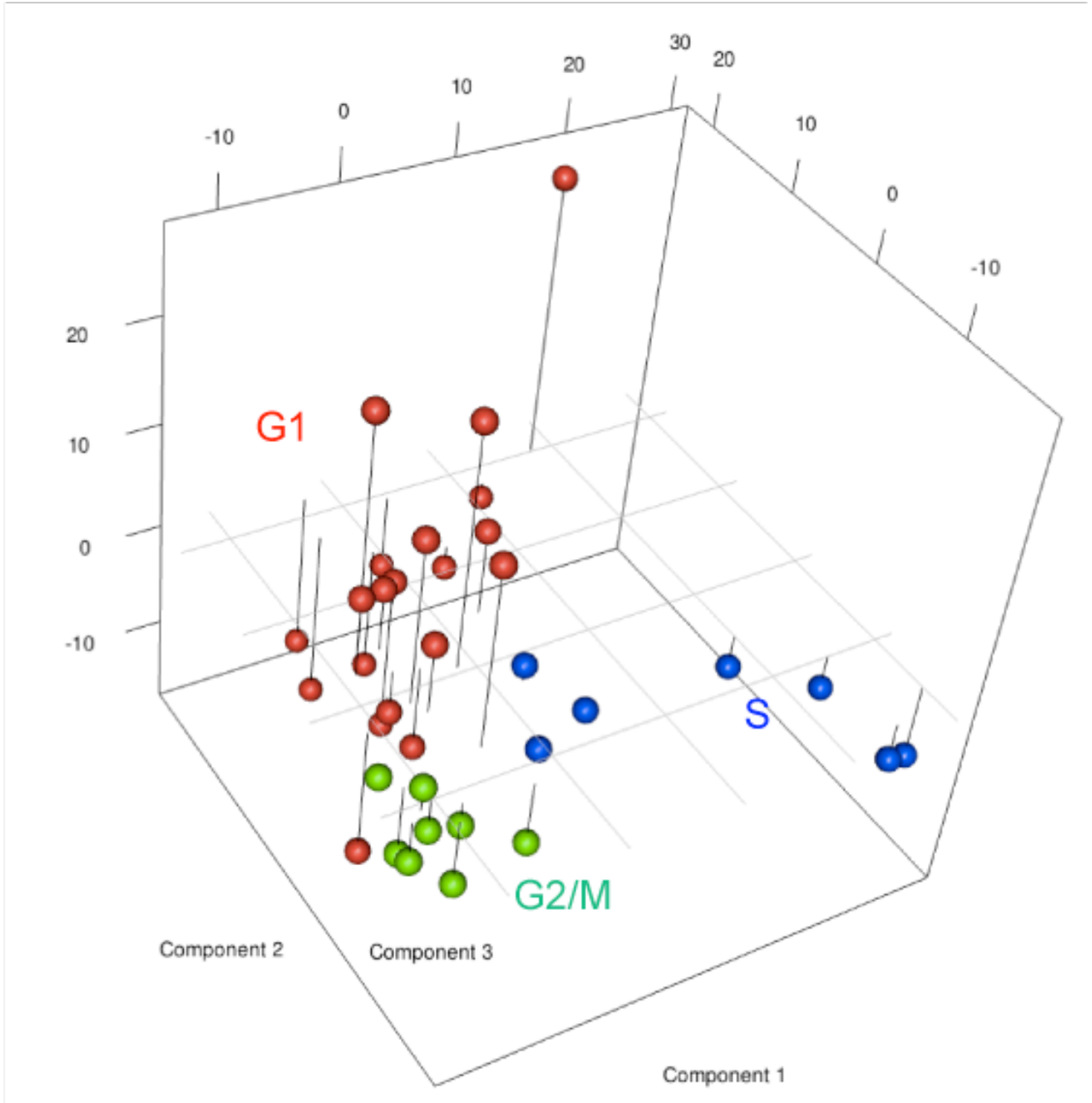
cellular heterogeneity in same culture condition (cell cycle)

single-cell sorting → 35 ES cells → single-cell RNA-seq

a



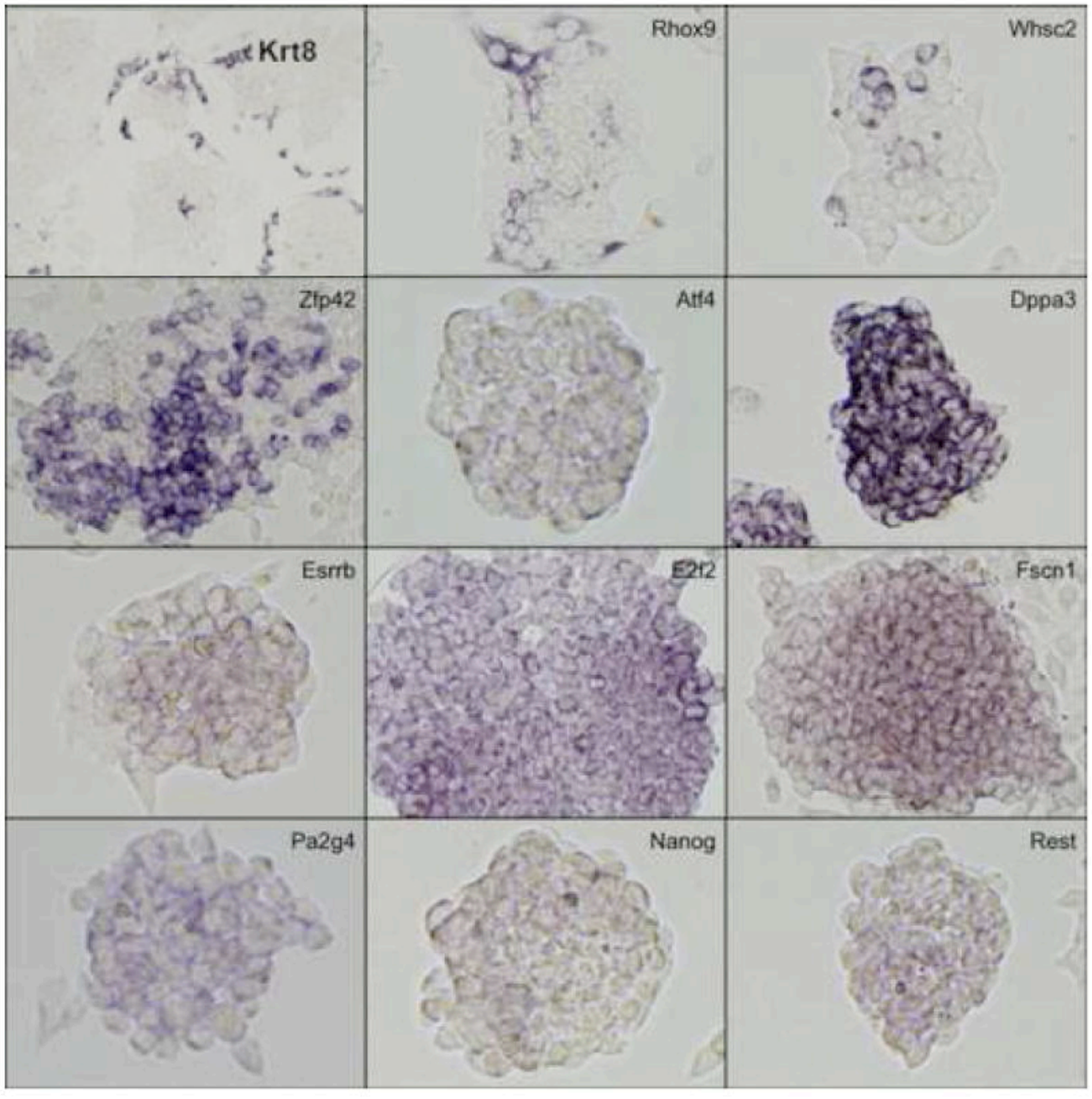
b



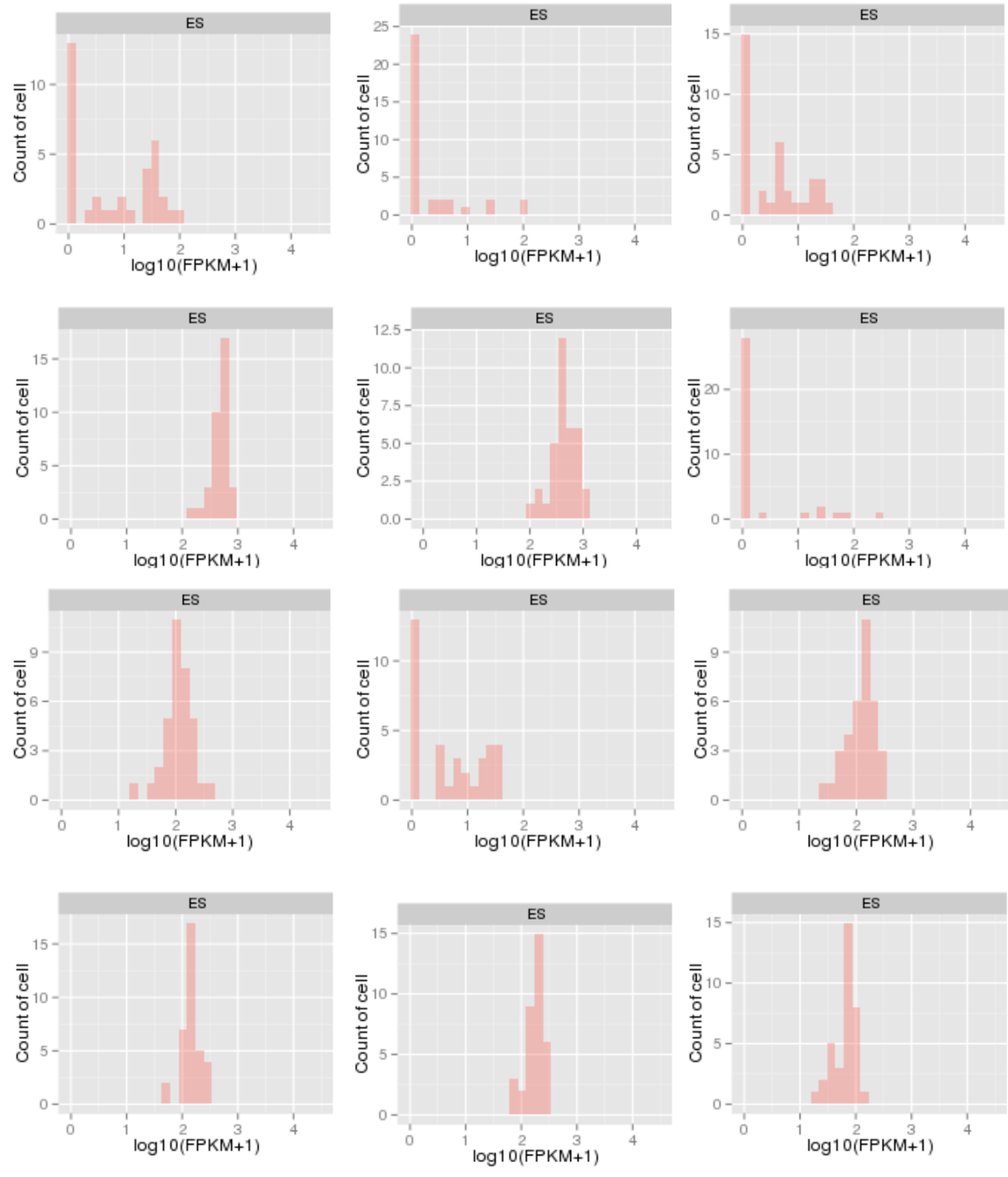
2. 同一細胞集団の発現ゆらぎを区別する (発現ゆらぎ)

cellular heterogeneity in same culture condition (gene expression variability)

in situ hybridization (Carter, MG. 2008)



Quartz-Seq (35 cells)



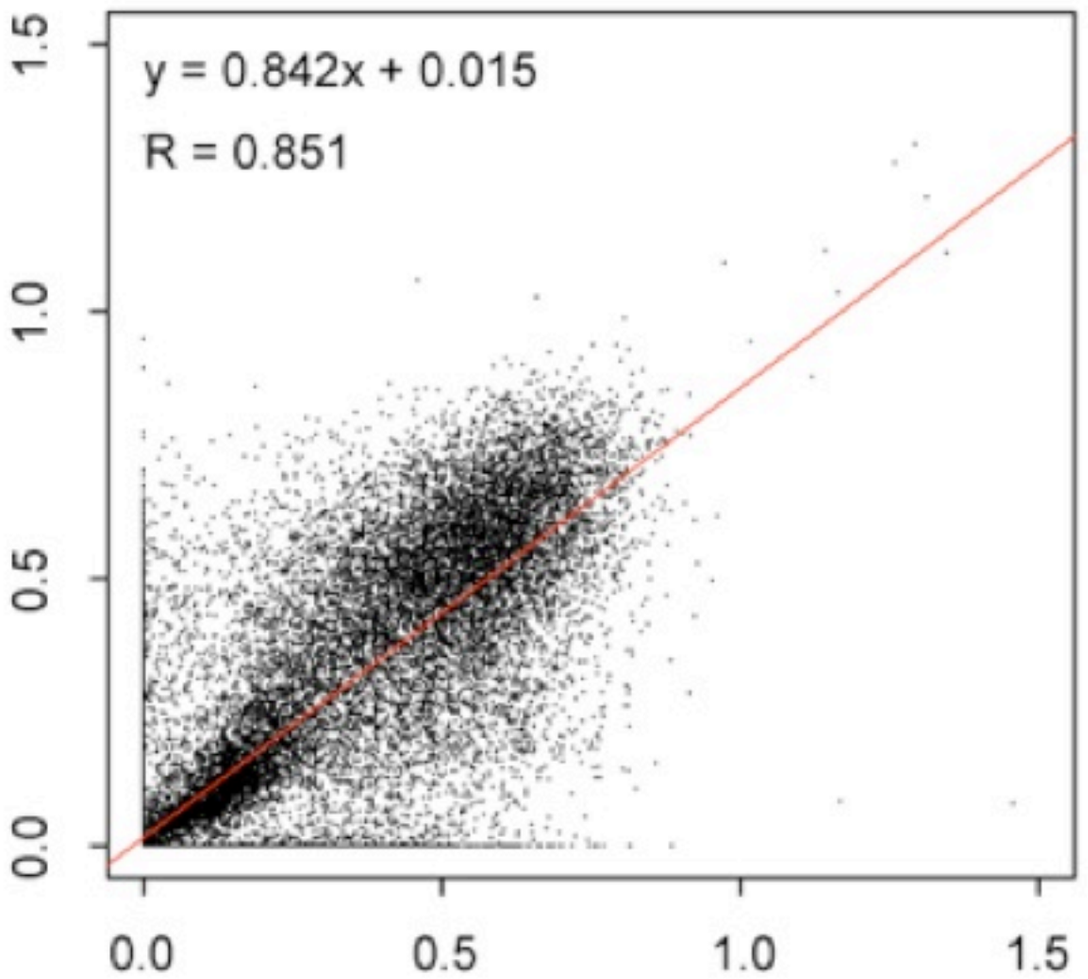
2. 同一細胞集団の発現ゆらぎを区別する (発現ゆらぎ)

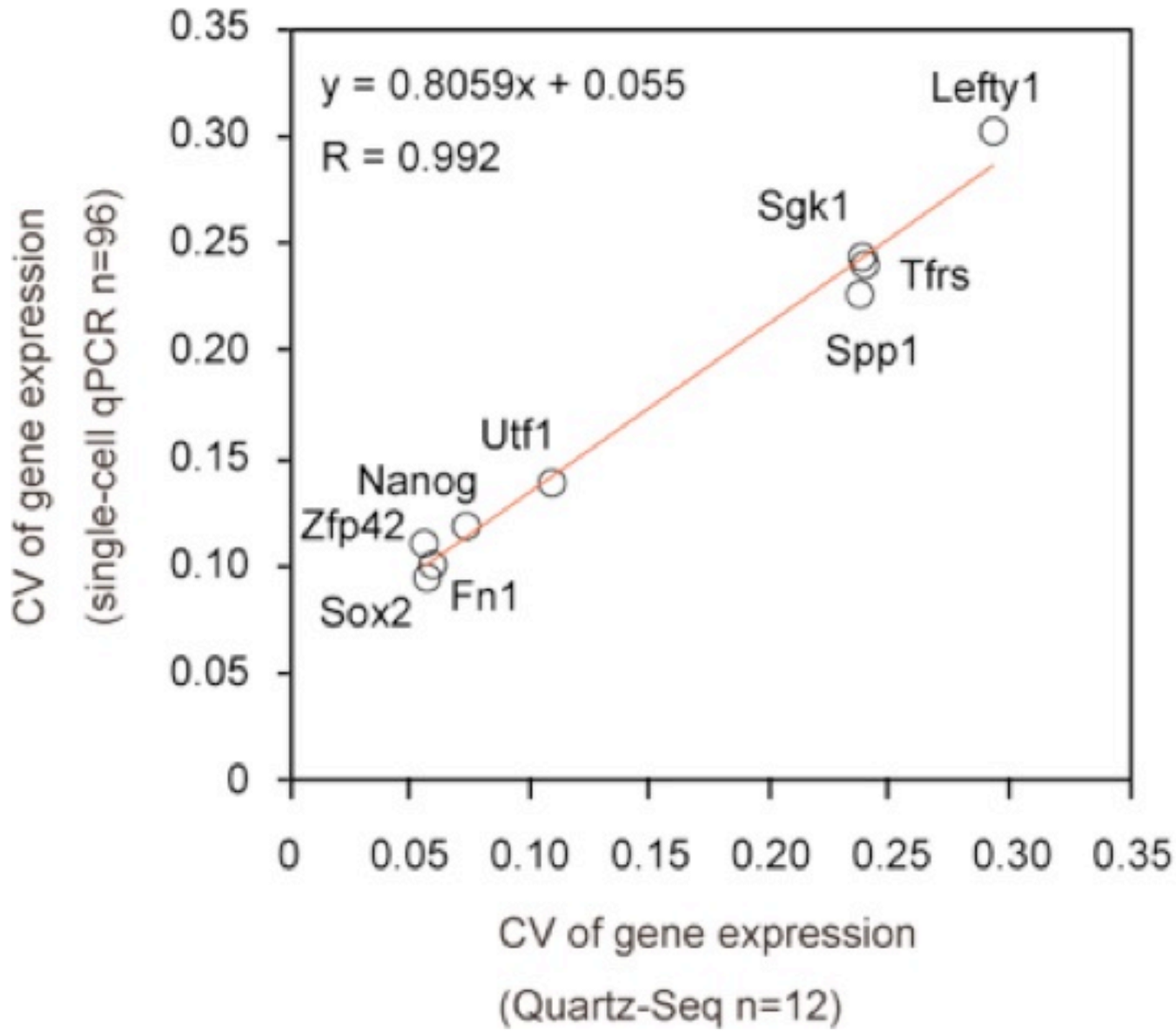
cellular heterogeneity in same culture condition (gene expression variability)

20 ES cells (G1 phase)

single-cell sorting ⟶ **single-cell RNA-seq**

F-test: 17,064 genes = reproducible





Non-amplification single-cell qPCR

gene expression variability

Single-cell qPCR
(96 cells)

Pooled sample

Single-cell qPCR
(96 cells)

Pooled sample

Over-representation analysis with Reactome

Enrichment of pathway in each gene cluster

まとめ

1細胞RNA-seq

1. 異なる細胞の種類を見分ける => 成功

cellular heterogeneity between different cell-types

2. 同一細胞集団の発現ゆらぎを区別する => 成功

cellular heterogeneity in same culture condition

http://bit.accc.riken.jp/protocols/

Quartz-Seqを導入する

その手順と注意点

0. 1細胞/微量RNA-Seqが本当に必要なのか検討する

50細胞程度でRNA-Seqしたほうがずっときれいなデータが取れる

戦略面での検討:
a. 細胞状態が連続的に変化し、さまざまな細胞状態が、細胞集団に含まれている場合 (振動現象、ゆらぎなど)
b. 細胞状態を特定するマーカーがほどんどわかっていない場合

技術面での検討:
1細胞あたりのRNA量を定量すること

Quartz-Seqを導入する

その手順と注意点

1. Quartz-Seqの導入

プロトコルの細部に至るまで意味があるので、条件を変えないこと

- 細胞集団のRNA-Seqのデータを取っておく
- 1細胞相当まで希釈した人工サンプルを作り、それを使って導入する
- cDNAの泳動像と収量を確認する
- qPCRとシーケンスで定量し, 相関係数とCV、検出遺伝子数で性能・安定性の評価をする

2. 細胞の採取

- 1細胞を採取し、細胞を融解する条件を作っておくこと
- Qaurtz-Seqの結果を評価する実験系を持つこと

Quartz-Seqを導入する

その手順と注意点

3. 検証系の立ち上げ

- Qaurtz-Seqの結果を評価する実験系を持つこと. RNA FISH, 1細胞qPCR

4. シーケンス計画

- 4-8 cells/lane, 2000万リード以上
- 20細胞以上
- Quartz WTAは1反応が500円
- Quartz-Seq 用のライブラリ作製法 LIMprep を利用すること

5. データ解析

- プライマー配列をトリミングする
- 1コピー以下を考える必要はない

本日のメニュー

0.1 pg の mRNA をシーケンスする高精度なRNA-Seq法: Quartz-Seq

0. なぜ1細胞RNA-Seqが必要か?

1. 1細胞RNA-Seq: Quartz-Seqの原理と利点

2. Quartz-Seqの性能と他の手法との比較

3. Quartz-Seqの導入方法と注意点

http://bit.accc.riken.jp/

Acknowledgments

0.1 pg の mRNA をシーケンスする高精度なRNA-Seq法: Quartz-Seq

Quartz-Seq

Yohei Sasagawa, Hiroki Danno, Hiroki R. Ueda

Tetsutaro Hayashi (GRAS)

Sequencing

GRAS (RIKEN CDB)

RIKEN GeNAS

Grant

Cell Innovation Project (MEXT)

NGS 現場の会

Next Generation Sequencer Field
次世代シーケンサー

第三回研究会
The third meeting

2013年
9月4日(水)5日(木)

神戸国際会議場
Kobe International Conference Center

事前申込
Pre-registration required

http://ngs-feld.org