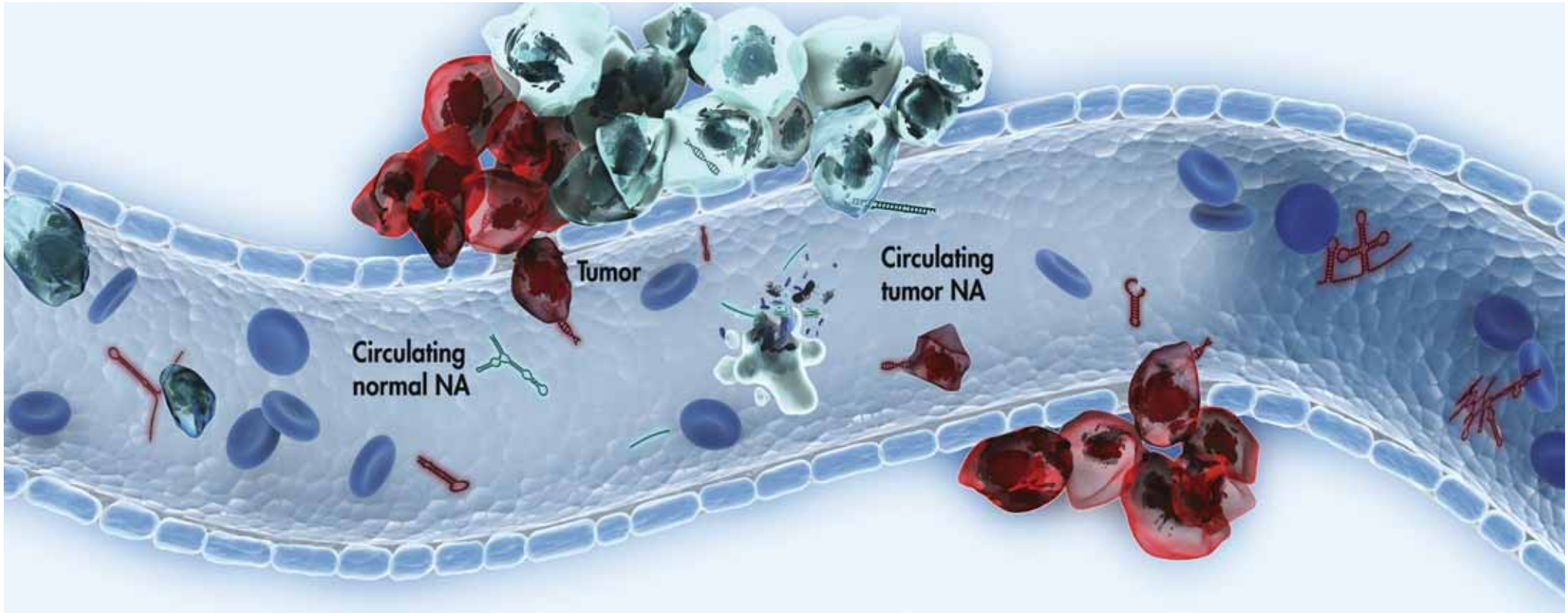


血漿中のmiRNAバイオマーカー探索からPathway 解析までのご紹介

— Liquid Biopsy の実用化に向けて —

株式会社キアゲン　北野 敦史

ゴール

これまで、Circulating miRNA **バイオマーカー候補と疾病や症状との相関は高いが、メカニズムは明らかになっていない報告が見られる**

Circulating miRNA **バイオマーカー候補の疾病や症状との関係を結び付ける事例と、**QIAGEN **の有用なツールを紹介する**

文献事例

PLoS One. 2014 Apr 2;9(4):e93297.

Concordant Changes of Plasma and Kidney MicroRNA in the Early Stages of Acute Kidney Injury: Time Course in a Mouse Model of Bilateral Renal Ischemia-Reperfusion

Melissa A. Bellinger[1], James S. Bean[1], Melissa A. Rader[1], Kathleen M. Heinz-Taheny[2], Jairo S. Nunes[3], Joseph V. Haas[4], Laura F. Michael[1], Mark D. Rekhter[1]*

1 Cardiometabolic Diseases and Diabetic Complications, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, United States of America, **2** Investigational Pathology, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, United States of America, **3** Covance Laboratories, Greenfield, Indiana, United States of America, **4** Statistics-Discovery/Development, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, United States of America

虚血再灌流障害モデルマウスのタイムコース実験の文献事例

早期の急性腎障害 (AKI) **モデルマウスにおいて、血漿と腎臓で一致する発現変動の** microRNAs **の中から、急性腎障害に対して関係の高い**Circulating miRNA Marker **候補が得られた**

文献の work flow

Sample prep → miRNA PCR Array → **発現が変動した** miRNAs → mRNA target **予測** → mRNA target **の** Pathway**解析** → Validation

Circulating miRNA Biomarker **候補** → **急性腎障害**

急性腎不全と虚血再灌流障害モデル動物

【急性腎不全】
虚血、薬剤、エンドトキシンショックなどの原因によって腎機能が急速に低下した状態

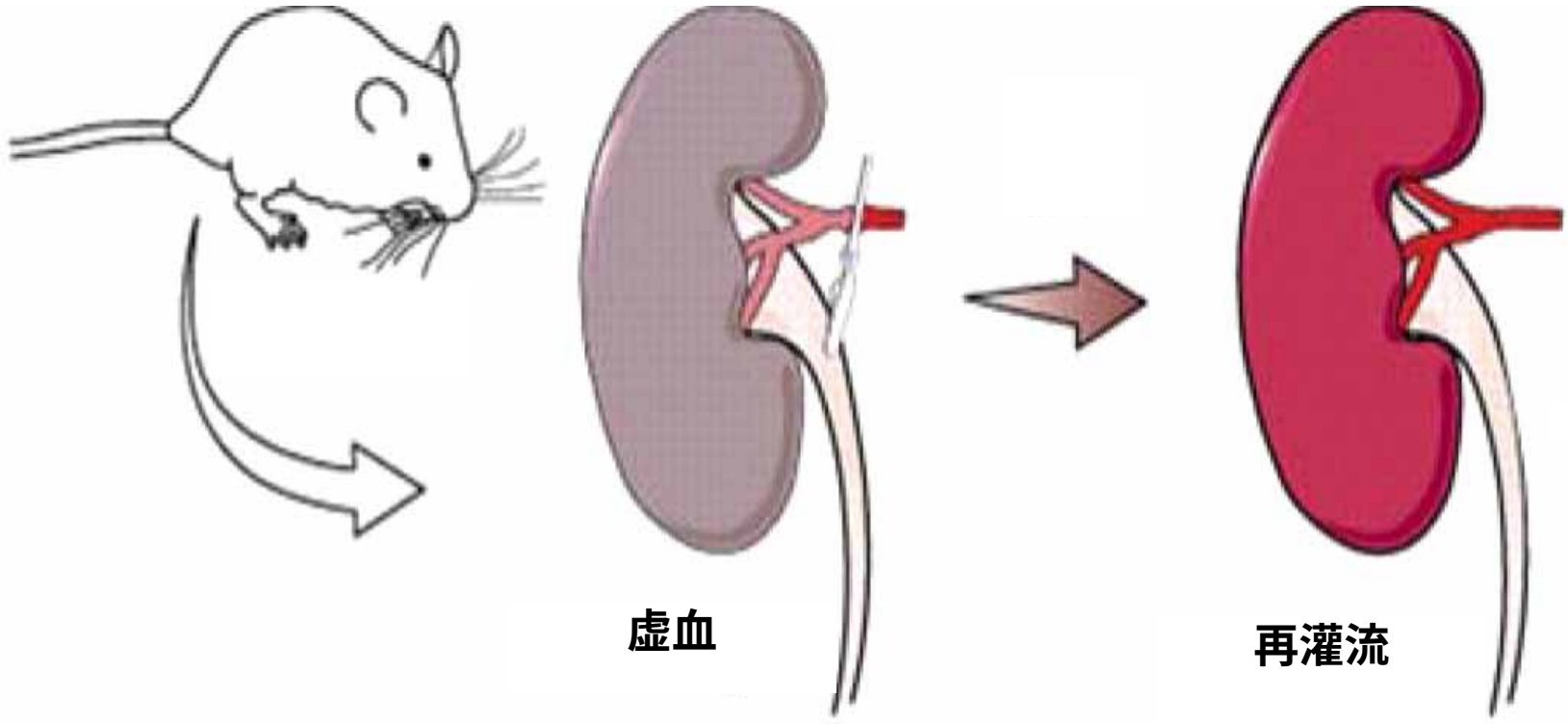
【急性腎不全モデル】
虚血再還流、重金属、各種薬物などによる腎機能低下モデルが用いられている

日薬理誌（*Folia Pharmacol. Jpn.*）**131**，37〜42（2008）

【虚血再灌流障害】
虚血状態にある臓器，組織に血液再灌流が起きた際に，その臓器・組織内の微小循環において種々の毒性物質の産生が惹起され引きおこされる障害をいう。

日本救急医学会・医学用語解説集

虚血再灌流モデル動物



Am J Physiol Renal Physiol 295: F310–F314, 2008. より改変

虚血再灌流障害を起こして急性腎不全のモデルとする

miRNA 精製

Sample prep → miRNA PCR Array → 発現が変動した miRNAs → mRNA target 予測 → mRNA targetの Pathway解析 → Validation

モデル Mouse
Control Mouse

→ Kidney → miRNeasy Kit

→ Plasma → miRNeasy Serum/Plasma Kit

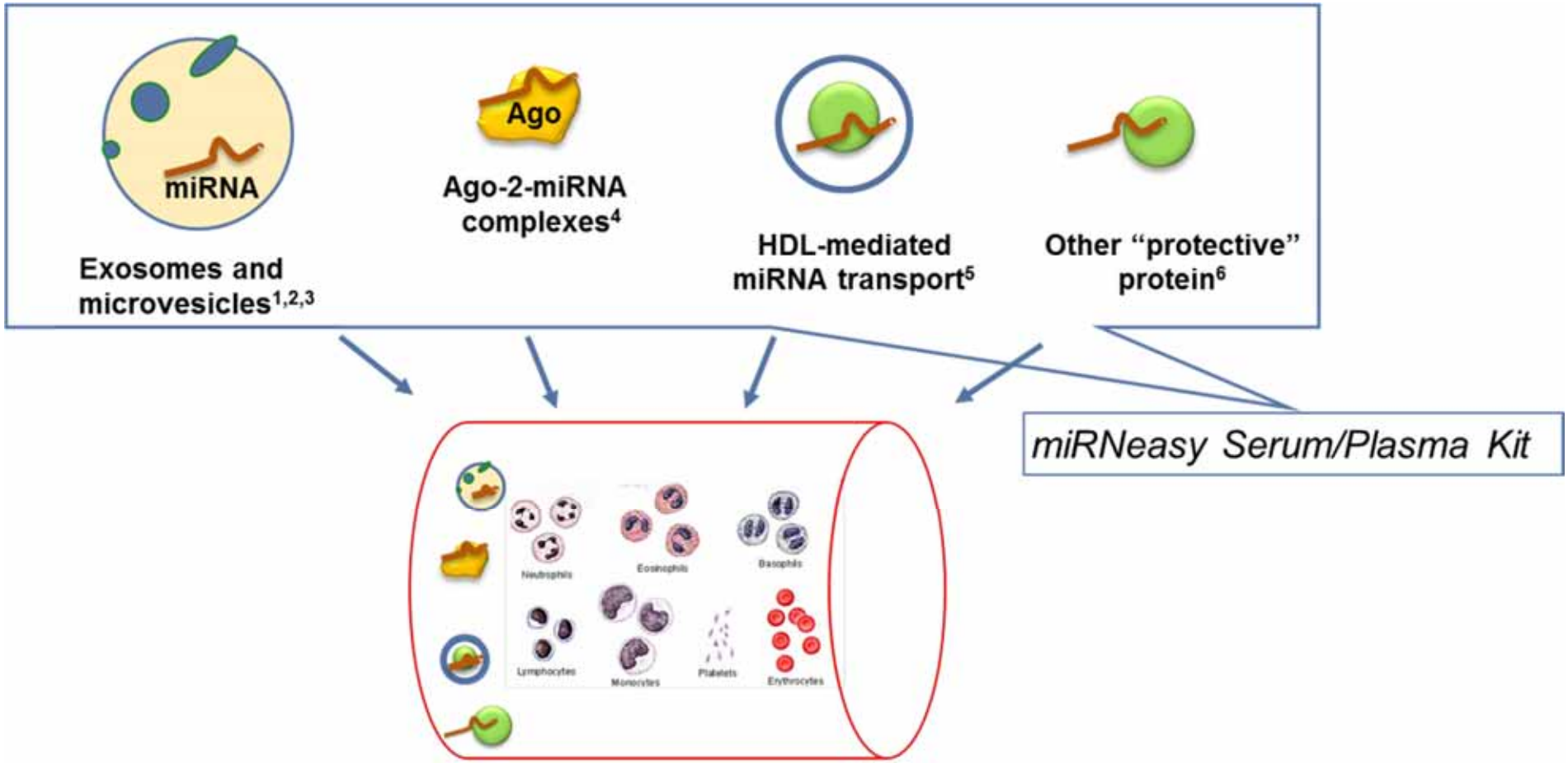
MicroRNA Profiling

Right kidneys were homogenized while still frozen using a Tissuemiser (Fisher, PA). 35 mg of the homogenate was used in subsequent isolation steps. Total RNA (including microRNA) was isolated from kidneys using miRNeasy kit (Qiagen, CA) according to manufacturer's protocol. Kidney RNA concentration and quality was assessed using the NanoDrop 1000 and the 2100 Bioanalyzer (Agilent, CA).

Total RNA (including microRNA) was isolated from EDTA plasma (~100 ul) using miRNeasy Serum/Plasma kit (Qiagen) according to manufacturer's protocol. C. elegans miR-39 miRNA mimic was spiked in at the beginning of isolation procedure for normalization purposes.

Plasma からのRNA 精製
センチュウ miR-39 を
外部Control としてspike

細胞外で安定なmiRNA



miRNA は細胞外で、タンパク質と複合体形成や小胞に内包されており、安定

1) Valadi, H., et.al.,(2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat Cell Biol **9**:654-659
2) Hunter MP et. al., (2008) Detection of microRNA Expression in Human Peripheral Blood Microvesicles, PLoS ONE **3**:e3694
3) Kosaka, N et. al (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells, J Biol Chem **285**: 17442-17452
4) Arroyo, JD et. al., (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc. Natl. Acad. Sci **108**: 5003-5008
5) Vickers, KC., et. al., (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol **13**:423
6) Wang K, Zhang S, Weber J, Baxter D, Galas DJ.(2010) Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res. 2010 Nov 1;**38**(20):7248-59.

miRNA 発現 Profiling

Sample prep → **miRNA PCR Array** → 発現が変動した miRNAs → mRNA target 予測 → mRNA targetの Pathway解析 → Validation

モデル Mouse
Control Mouse

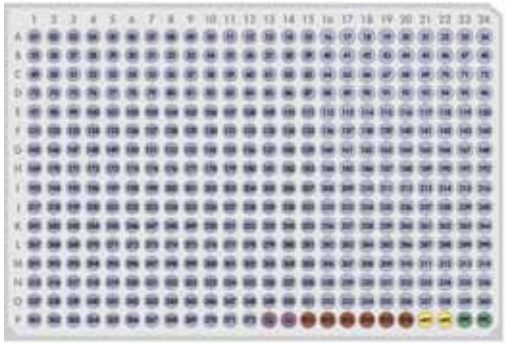
→ Kidney → miRNeasy Kit →
→ Plasma → miRNeasy Serum/Plasma Kit →

miScript PCR Array

Equal volumes (plasma) or amount (kidney) of RNA were pooled within each group. Mature microRNA was reverse transcribed using miScript II RT kit (Qiagen) according to manufacturer's protocol. RT PCR was performed on the cDNA using Mouse miRNome miScript miRNA PCR Arrays (v16.0

assays (Qiagen). Fold changes were calculated as described above. C. elegans miR-39 and SNORD61 were used as housekeeping genes for plasma and kidneys respectively.

miScript PCR Array (miRBase v16) miRNAs に対するPrimer が スポット済みqPCR Plate

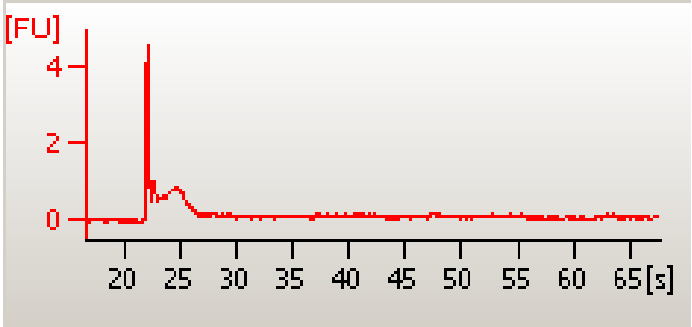


SYBR Green **で検出**

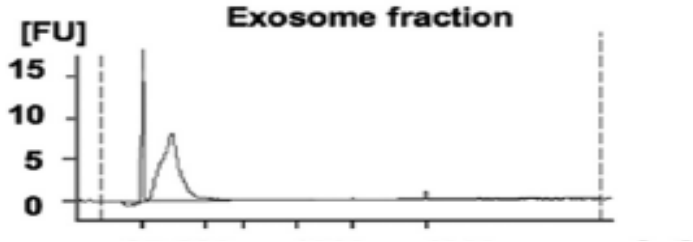
miRNeasy Serum/PlasmaとmiScript PCR Arrayの組み合わせのアドバンテージ

✓ 細胞外RNA は、rRNA を含んでいない

Serum RNA の泳動例（QIAGENデータ）



培養細胞上清のExosome RNA (文献の泳動例)



Kosaka et al., **J. Biol. Chem.,** 2010; 285: 17442

✓ 細胞外RNA は、NanoDrop などの分光光度計にて定量出来ない*
✓ 多くの miRNA microarray や qPCR は、細胞内 RNA（rRNA含む）でprotocolが最適化されている

*McDonald JS, Milosevic et.al: Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 2011 Jun;57(6):833-40.

miRNeasy Serum/PlasmaとmiScript PCR Arrayの組み合わせのアドバンテージ

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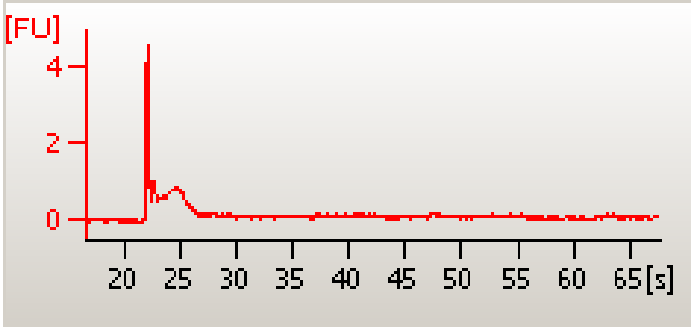
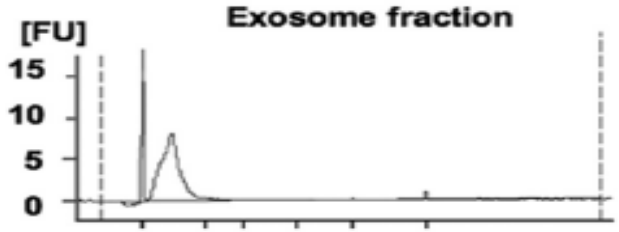
Serum RNA の泳動例（QIAGENデータ）



培養細胞上清のExosome RNA (文献の泳動例)



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*McDonald JS, Milosevic et.al: Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 2011 Jun;57(6):833-40.

Table 4. Reverse-transcription reaction components

Component	Volume/reaction
5x miScript HiSpec Buffer	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA (added in step 3)	Variable (see Table 3 for recommendations)*
Total volume	**20 µl**

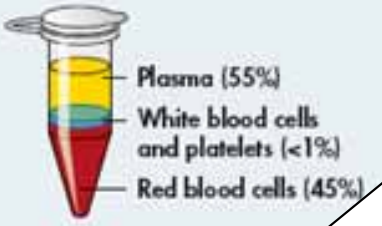
* If RNA was prepared from 100–200 µl serum or plasma using the miRNeasy Serum/Plasma Kit, up to 9 µl RNA prep can be added to the reverse-transcription reaction (sufficient for 6 x 384-well plates or 24 x 96-well plates/Rotor-Discs).

- miRNeasy Serum/Plasma Kit と miScript PCR Array の組み合わせはSerum / Plasma などの体液で検証されている
- ユーザーは、検証済みのSample 量とRNA 量を使用することができる

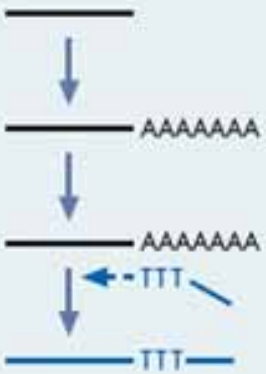
miRNeasy Serum/PlasmaとmiScript PCR Arrayの組み合わせのアドバンテージ

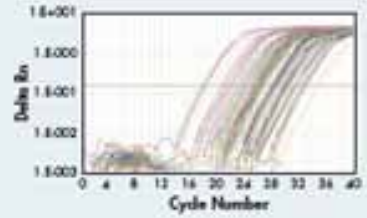
assays (Qiagen). Fold changes were calculated as described above. C. elegans miR-39 and SNORD61 were used as housekeeping genes for plasma and kidneys respectively.

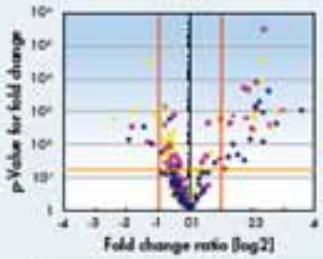
miRNeasy
+
Spike-in control

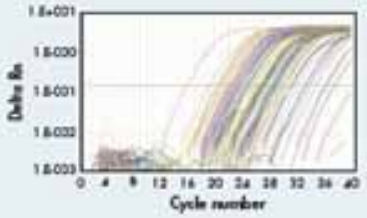


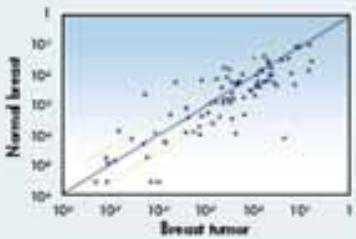
オプション:
センチュウ miR-39
を添加

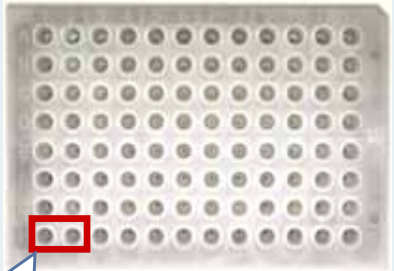












センチュウ miR-39

センチュウ miR-39 を 外部コントロールとして使用し、RNA 精製の回収率を補正できる

► **信頼性の高い結果が得られる**

発現が変動した miRNAs

Sample prep → miRNA PCR Array → **発現が変動した miRNAs** → mRNA target 予測 → mRNA targetの Pathway解析 → Validation

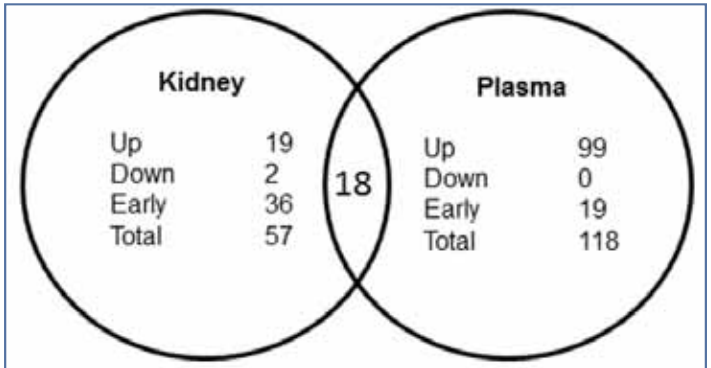


Figure 4. Pattern of microRNA modulation in kidneys and plasma of mice undergoing ischemia-reperfusion. While multiple microRNA species were modulated in the kidneys and in plasma after ischemia-reperfusion, 18 microRNAs were modulated in both compartments in a time-dependent manner. **Up**, increased expression; **Down**, decreased expression; **Early**, modulated at 3 hours and returned to the Sham level at 24 hours.
doi:10.1371/journal.pone.0093297.g004

the same direction (Figure 4). In order to identify miRNAs likely arising from the kidney that could serve as plasma-borne biomarkers of renal injury, we focused our analysis on miRNAs that were regulated in both kidney and plasma.

モデルマウスのKidney 由来するPlasma 中の マーカーを得るため、両方で同じ発現をするmiRNAs に注目

plasma is also unclear. Elevation of certain microRNAs in plasma may represent a result of renal cell death associated with leakage of cytoplasmic components. Alternatively, microRNAs can be

腎障害による細胞死で、細胞内 miRNA がPlasma に漏れ出た可能性

Kidney とPlasma の両方で同じ発現を示した miRNAs

Sample prep → miRNA PCR Array → **発現が変動した miRNAs** → mRNA target 予測 → mRNA targetの Pathway解析 → Validation

Table 2. MicroRNAs concordantly modulated in kidneys and plasma (pooled sample results).

miR	Kidney			Plasma		
	3 hr	6 hr	24 hr	3 hr	6 hr	24 hr
mmu-miR-714	3.21	2.72	6.23	1.59	5.52	13.72
mmu-miR-1188	1.55	2.50	4.13	1.69	8.20	9.20
mmu-miR-1897-3p	3.72	5.82	11.12	7.88	4.12	13.12
mmu-miR-877*	1.38	2.28	2.90	1.91	2.21	5.84
mmu-miR-3471	3.77	2.59	1.06	11.04	10.25	10.10
mmu-miR-1224	1.45	1.83	4.77	−1.23	4.23	9.14

► Kidney とPlasma の両方で発現量が上昇している、miR-1897-3p を marker 候補

miR-1897-3pのmRNA target予測

Sample prep → miRNA PCR Array → 発現が変動した miRNAs → **mRNA target 予測** → mRNA targetの Pathway解析 → Validation

Because miR-1897-3p had the greatest modulation, our goal was to identify its mRNA targets and evaluate possible regulation of pathogenic pathways by miR-1897-3p. The top two targets generated by mirBase v.19 were Lass4 and Nucks1 with Target Scores of 80 and 79 respectively [14–17]. TargetScan Mouse 6.2 identified Nucks1 as the top target (total context score = −0.53) closely followed by Lass4 (total context score = −0.43) when

miR-1897-3p のmRNA target をmirBaseやTargetScan を用いて予測

► miR-1897-3p の mRNA target 候補は、Lass4 と Nucks1

mRNA target の下流を解析

Sample prep → miRNA PCR Array → 発現が変動した miRNAs → mRNA target 予測 → **mRNA targetの Pathway解析** → Validation

Nucks1

MDM2

CLU	Apoptosis of renal epithelial tubular cells, Nephritis
AATF	Apoptosis of renal epithelial tubular cells
HIF1A	Nephritis
PDE4D	Nephritis, Renal failure
SKP2	Renal failure
TOP2A	Acute renal failure
GSTP1	Acute renal failure, proximal tubular toxicity
HSP90AA1	Proximal tubular toxicity
PSMA3	Proximal tubular toxicity

MME

LYN	Nephritis
PRKCD	Nephritis
IMPDH2	Nephritis, renal failure
AGT	Nephritis, renal failure, apoptosis of podocytes, glomerular, and proximal tubular cells, injury of kidney
EDN1	Renal failure, apoptosis of podocytes and gomerular cells, damage of tubular interstitium
CD44	Reperfusion injury of kidney
IL1b	Apoptosis of mesangial and glomerular cells, proximal tubular toxicity
HSPB1	Proximal tubular toxicity

Figure 6. Downstream targets of Nucks1. Pathway analysis was performed on Nucks1 (nuclear substrate 1) using Ingenuity Pathway Analysis. Downstream targets of Nucks1 appear to be involved ir

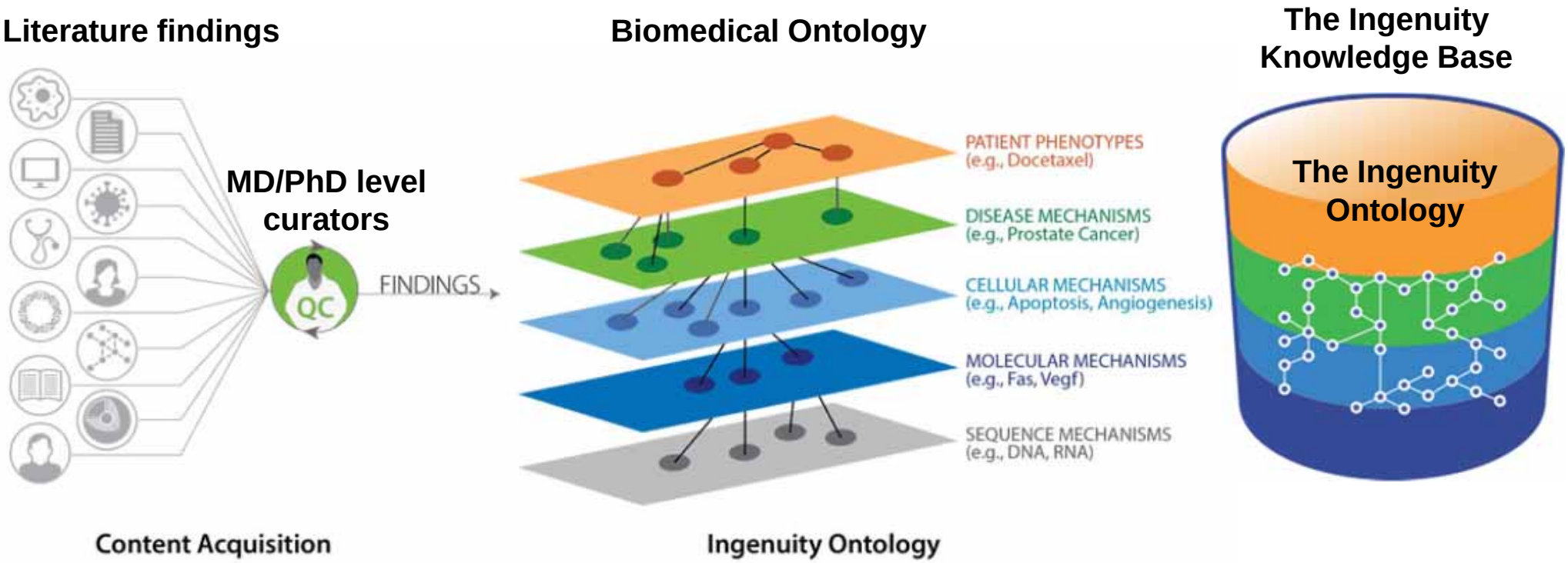
mirSVR score of −0.59 [21–22]. Pathway analysis was performed on Nucks1 (nuclear casein kinase and cyclin-dependent kinase substrate 1) using Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA). Indeed, downstream targets of Nucks1 are involved in renal injury, inflammation, and apoptosis (Figure 6).

miR-1897-3p の mRNA target 候補の下流の Pathway を Ingenuity Pathway Analysis (IPA) で解析した

► **Nucks1 の下流遺伝子の一部が、研究対象の疾病や症状と関連していることが分かる**

Ingenuity Pathway Analysis (IPA)

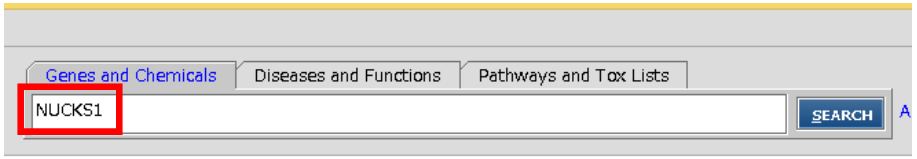
IPA のIngenuity ナレッジベースはタンパク質、遺伝子、複合体、細胞、組織、薬、疾患に関わる数百万にも及ぶ生物学的機能、相互作用に関する情報が15年もの間収集されたデータベースです

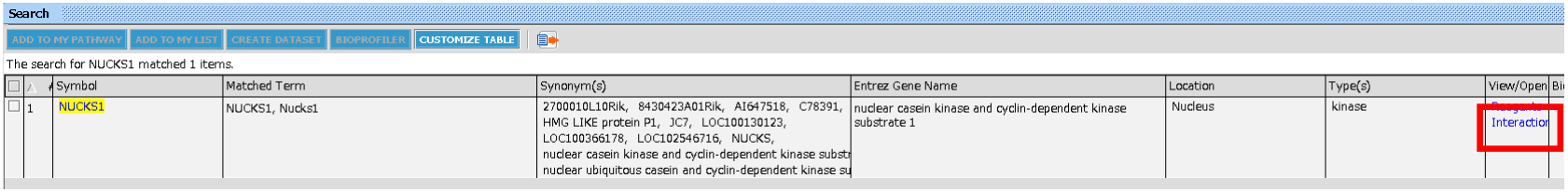


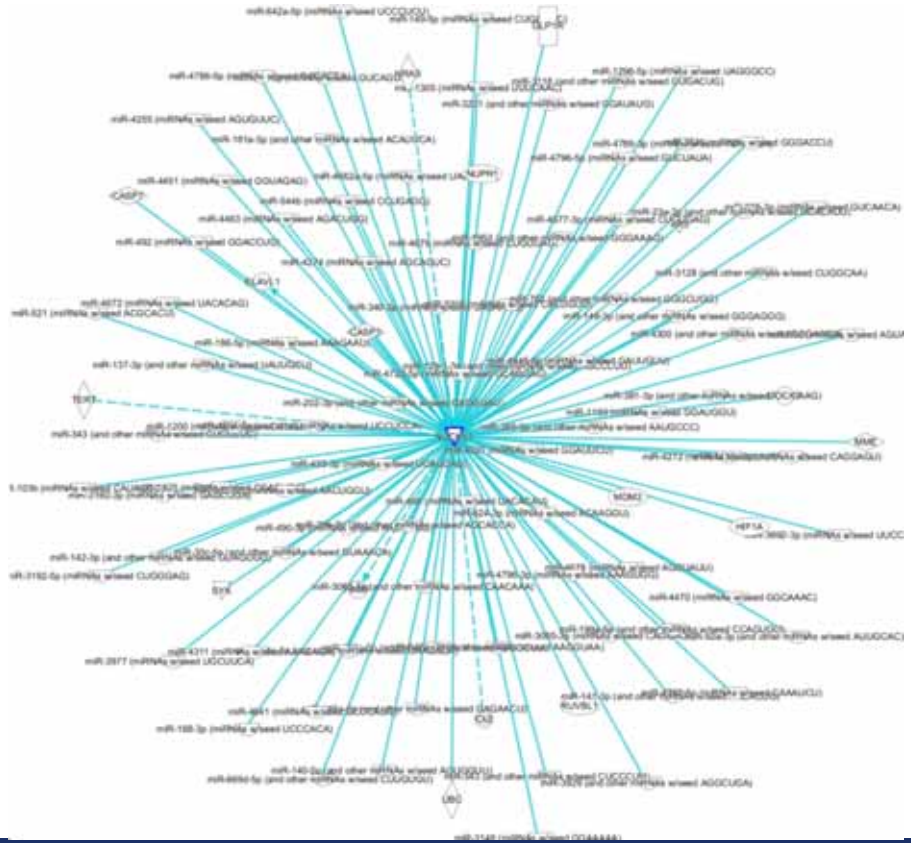
Ingenuity ナレッジベースを駆使した様々な活用方法

- ✓ Gene/Chemical、機能、Pathway のdata 検索
- ✓ Pathway の調査、仮説の作成
  どの分子が研究対象の分子 / 疾病と作用しているか
- ✓ データ解析

IPA によるNucks1の下流の遺伝子の探索



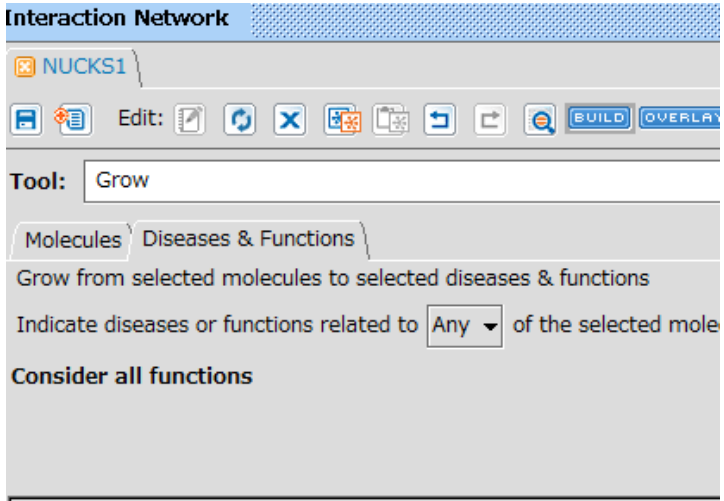




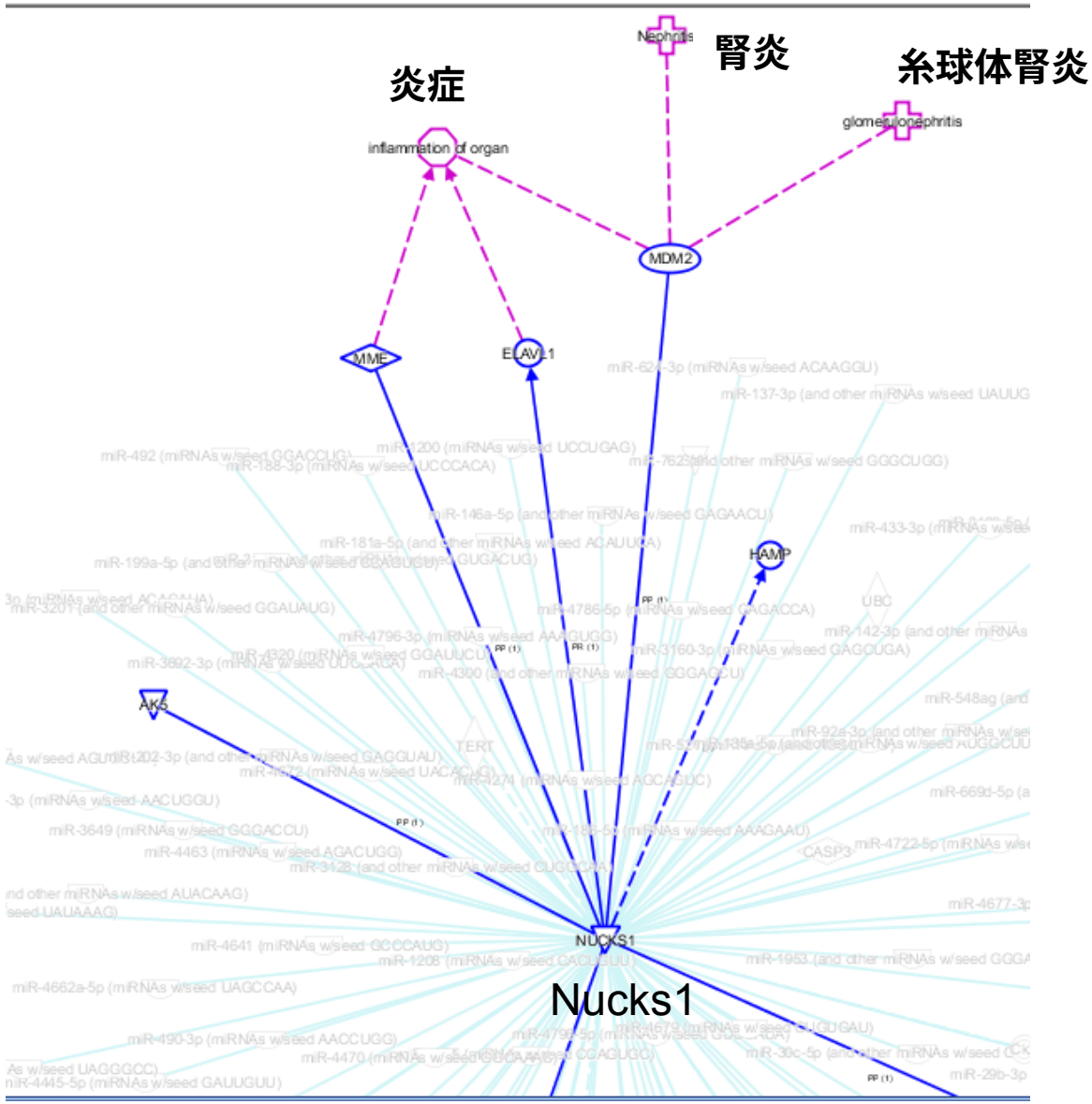
Nucks1 とInteraction の報告がある分子が表示

IPA によるNucks1の下流の遺伝子の探索

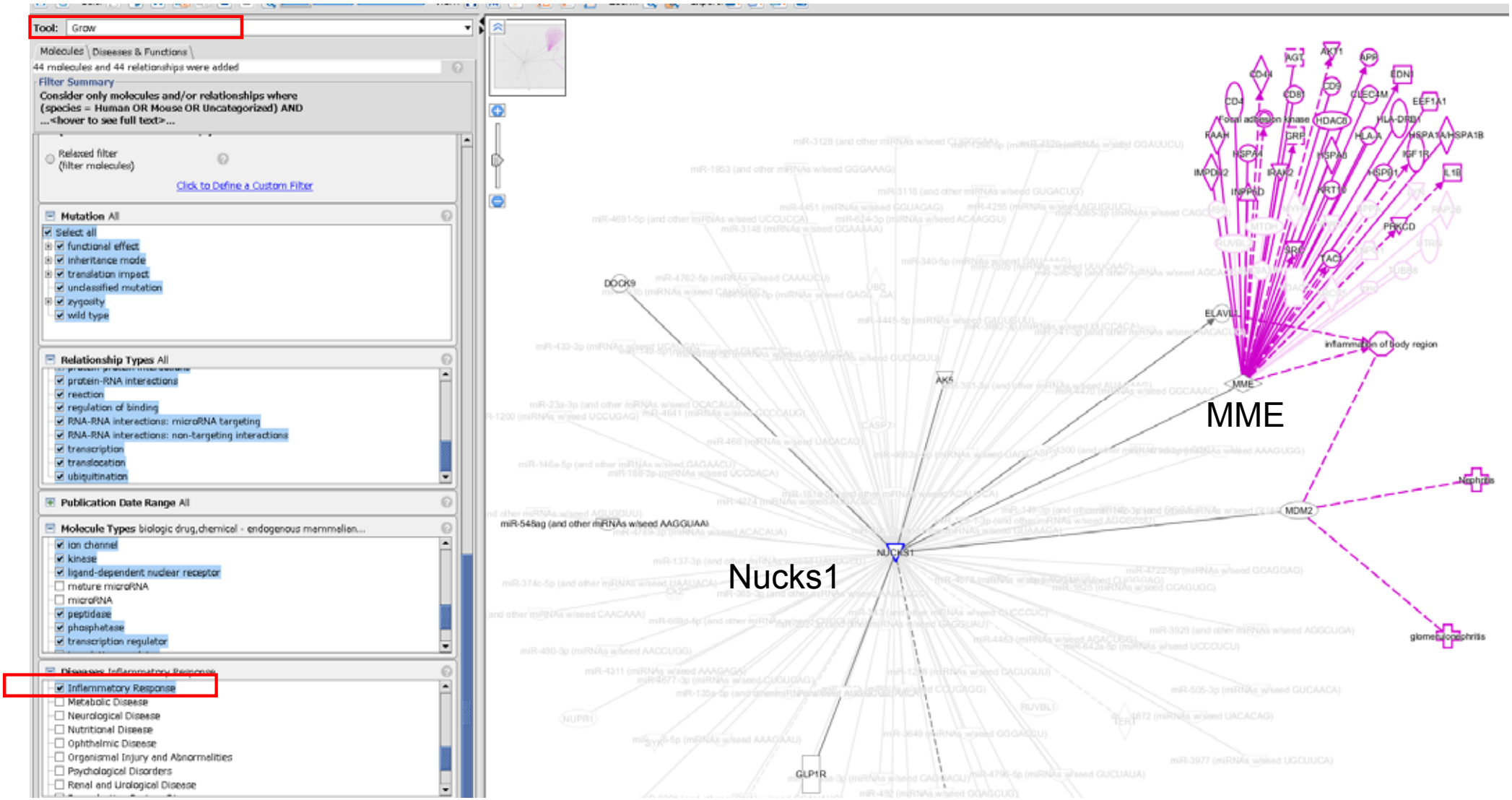
Nucks1 の上流の遺伝子を除いて、下流の遺伝子と研究対象の疾病との関連を表示



Diseases and Functions	p-value	Molecules
early stage cervical squamous cell	4.10E-23	miR-135a... ..
invasive cervical carcinoma	4.10E-23	miR-135a... ..
invasive squamous-cell carcinoma	1.13E-22	miR-135a... ..
cervical squamous cell carcinoma	7.61E-21	miR-135a... ..
cervical cancer	1.39E-18	miR-135a... ..
nonobstructive azoospermia	2.00E-17	miR-374c... ..
azoospermia	4.23E-17	miR-374c... ..
invasive carcinoma	1.63E-16	miR-135a... ..
class II lupus nephritis	1.46E-15	miR-642a-...
inflammation of body cavity	8.78E-14	miR-135a... ..
liposarcoma	3.08E-13	miR-146a-...
inflammation of body region	3.84E-13	miR-140-... ..
inflammation of organ	1.55E-12	miR-140-... ..
glomerulonephritis	2.50E-12	miR-642a... ..
squamous-cell carcinoma	3.17E-12	miR-135a... ..
Nephritis	3.44E-12	miR-642a... ..
angiocentric T-cell lymphoma	5.57E-12	miR-140-5...

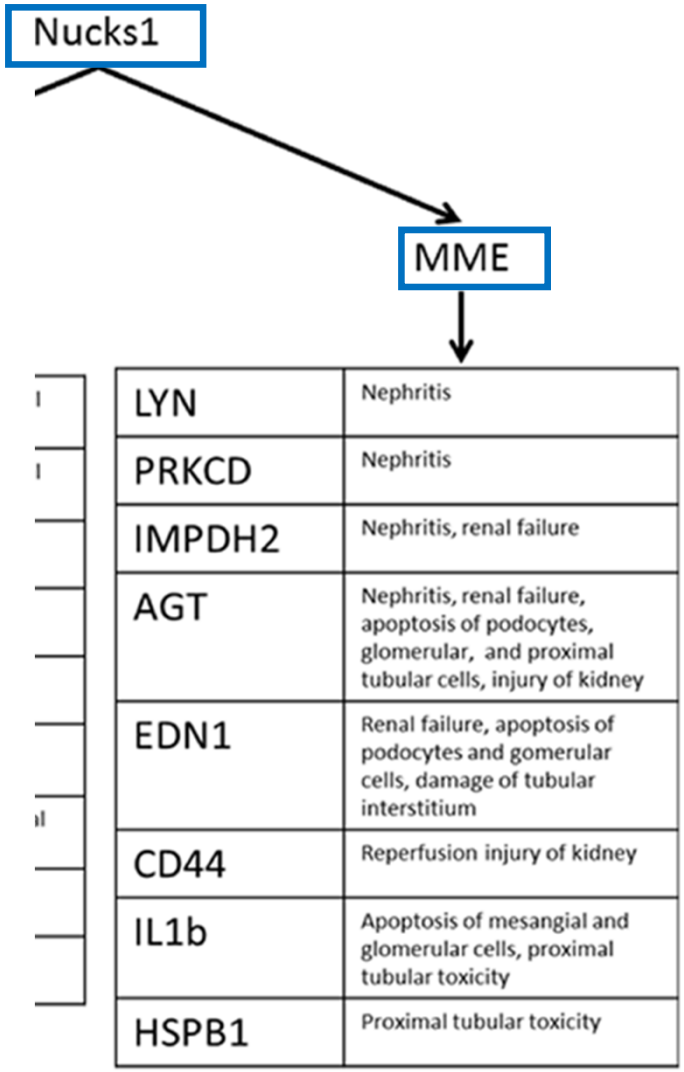


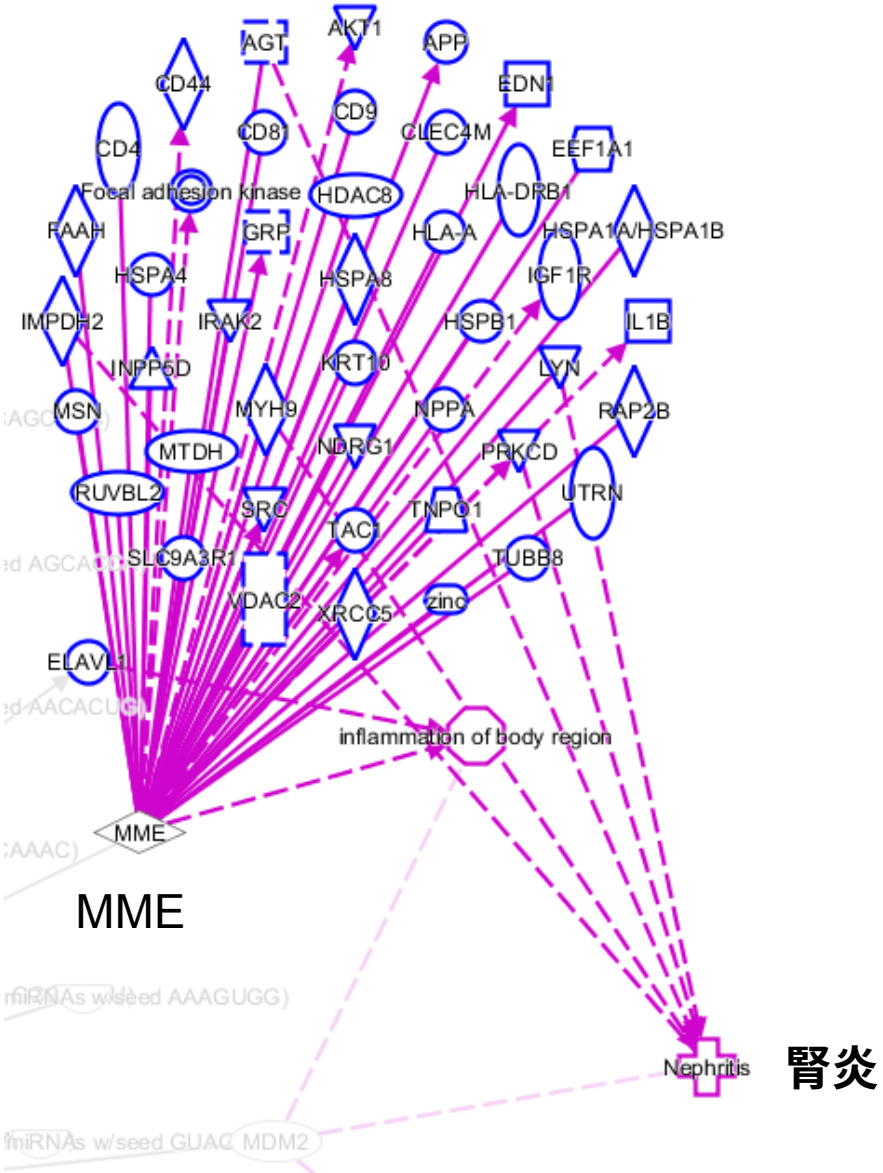
IPA によるNucks1の下流の遺伝子の探索



MME は、Nucks1 の下流の遺伝子のうちの1つ
MME の下流の遺伝子で研究対象の疾病や症状と関連する遺伝子のみ interaction を伸ばす

IPA によるNucks1の下流の遺伝子の探索





MME

- ▶ MMEの下流の遺伝子と、研究対象の疾病や症状との関連を見つけることができる
- ▶ miR-1897-3p → Nucks1 → 下流遺伝子 → 腎炎を含む疾病の関係が予測された

損傷したKidney 内で、Nucks1のdown-regulationを確認

Sample prep → miRNA PCR Array → 発現が変動した miRNAs → mRNA target 予測 → mRNA targetの Pathway解析 → Validation

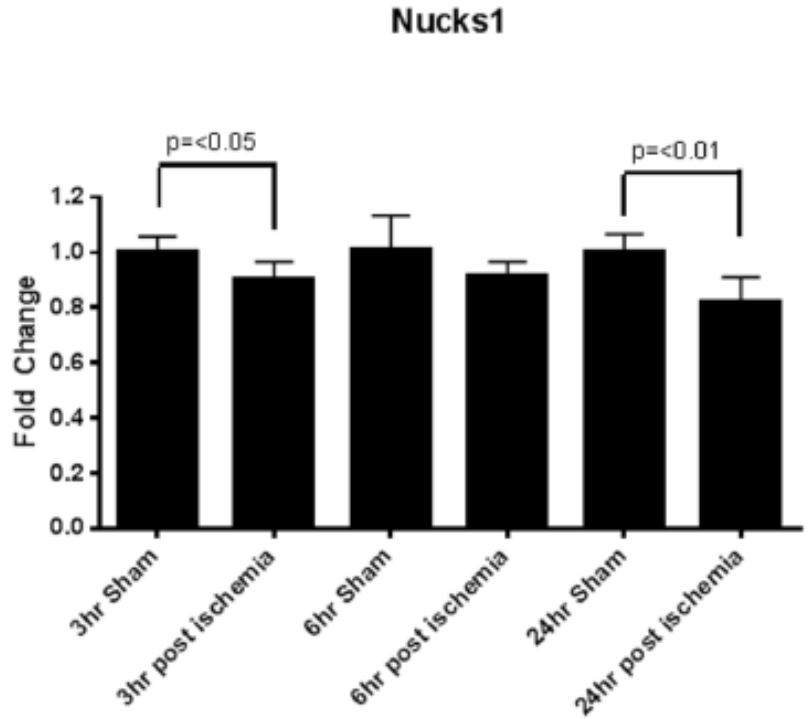


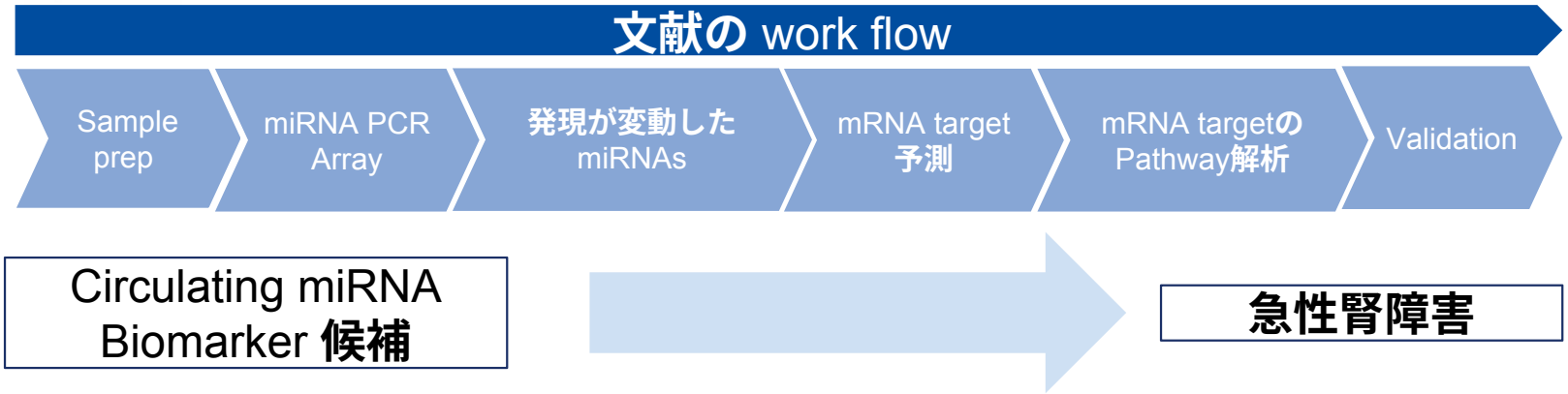
Figure 7. Nucks1 expression in the kidneys of mice with renal ischemia-reperfusion. Nucks1 gene expression was down-regulated

Experimentally, Nucks1 gene expression was down-regulated in the injured kidneys at all timepoints and reached significance at 3 and 24 hours (Figure 7). These findings suggest that miR-1897-3p may, in fact, serve as a marker of renal injury and contribute to progression of renal dysfunction following an ischemic event.

miR-1897-3p の target 候補の Nucks1 は、モデルマウスのKidney 内で 発現量が低下していた

- miR-1897-3p の発現量の増加により Nucks1 のdown-regulation が生じたと思われる
- Nucks1 はmiR-1897-3p のTarget と思われ、Nucks1 のdown-regulation により腎損傷に関連する下流遺伝子が影響を受けたと思われる

文献事例の work flow

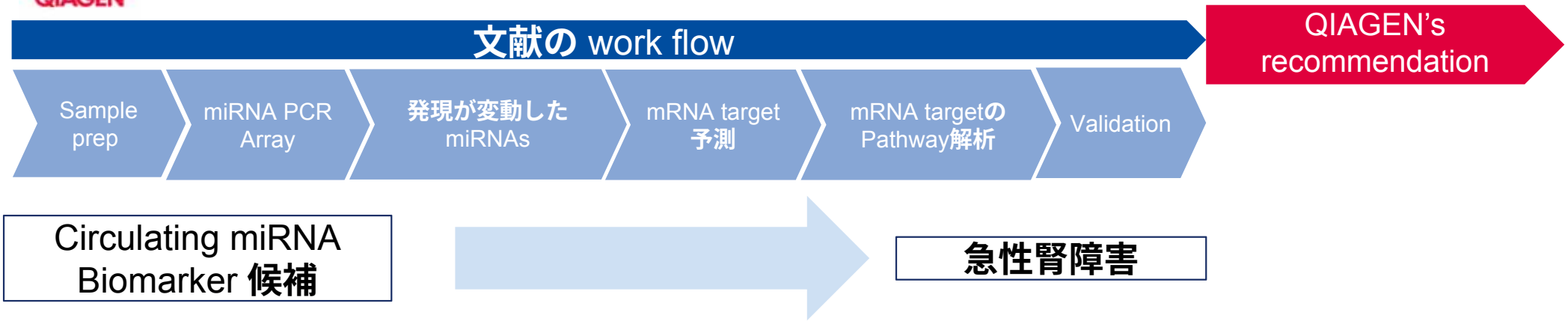


幾つかの疑問が残っているのでは？

1. Nucks1 は本当に miR-1897-3p のtarget か?

2. 検証は、Nucks1 がdown-regulation されたのを確認しただけで充分？
   Nucks1 の下流の遺伝子の発現も解析するべきでは？

文献事例の work flow



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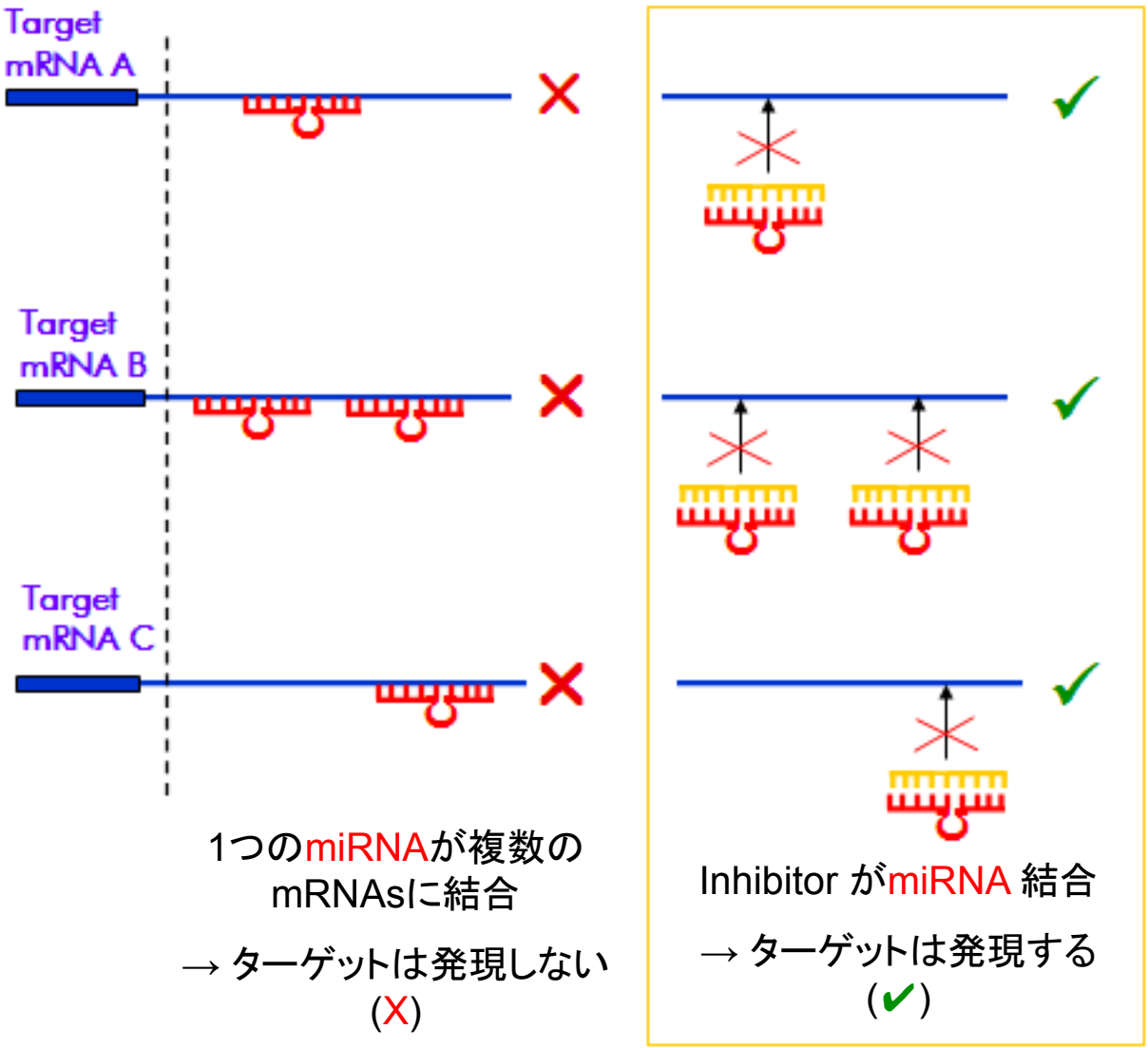
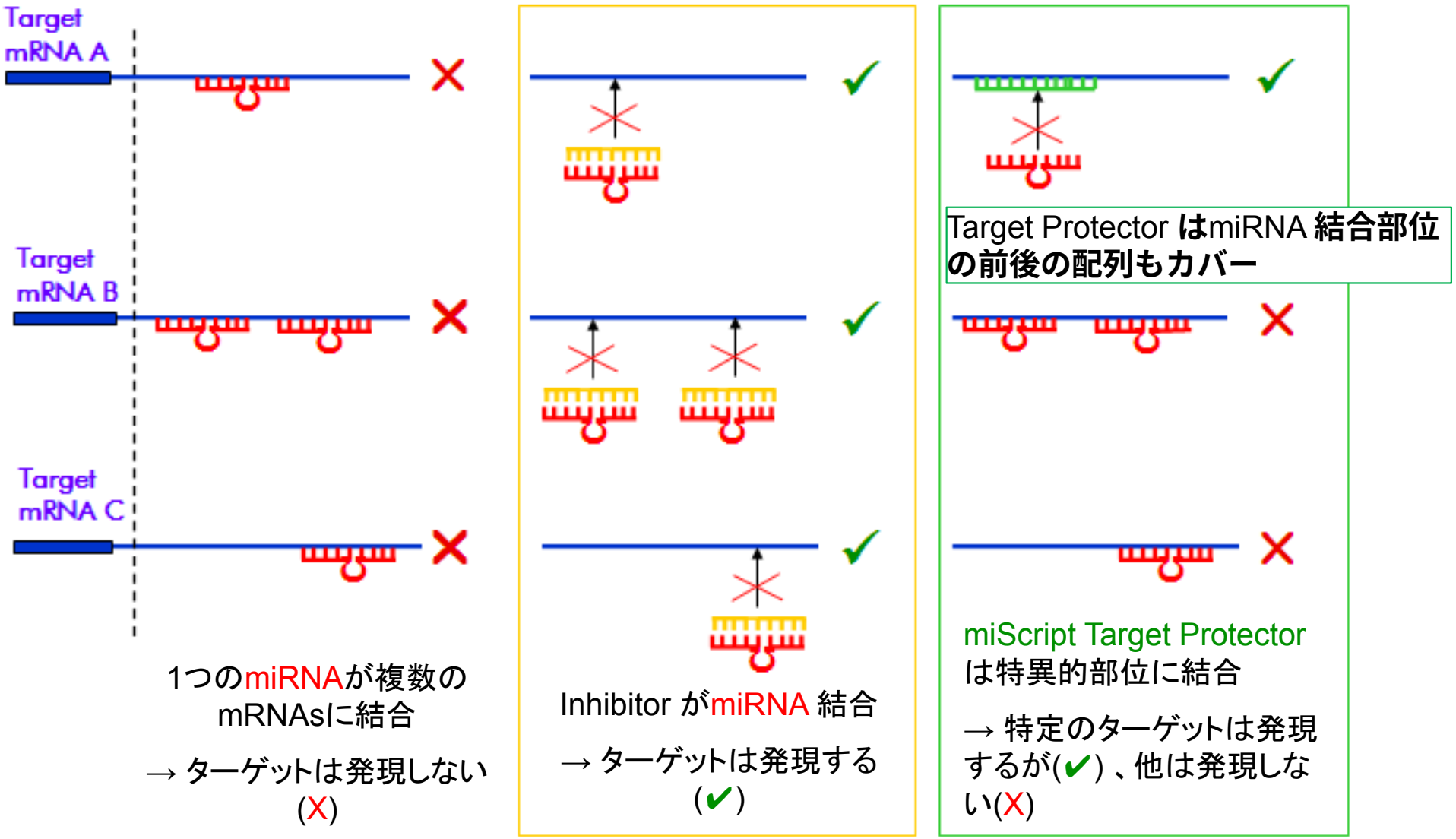
   Nucks1 の下流の遺伝子の発現も解析するべきでは？

miRNA Target Validation : miRNA mimic / Inhibitor の課題



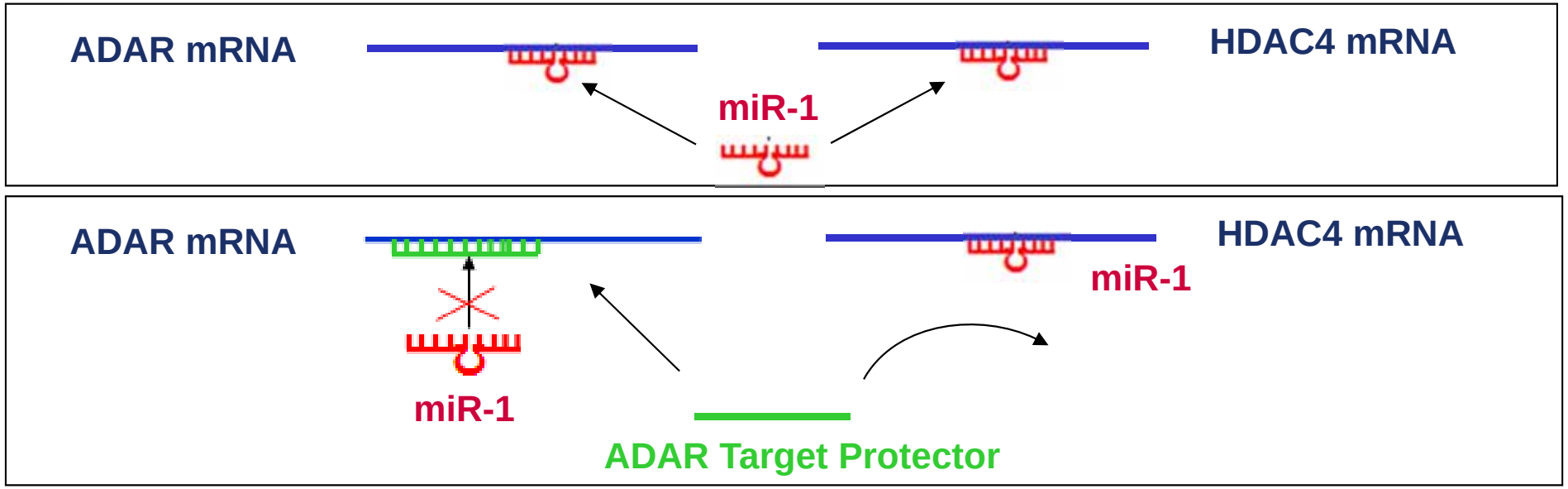
1つのmiRNAが複数種類のmRNAsに結合するため、miRNAの過剰発現や阻害は全てのmRNA targetに影響。フェノタイプやタンパク質レベルでの結果が複雑でありウエスタンブロットでは不充分

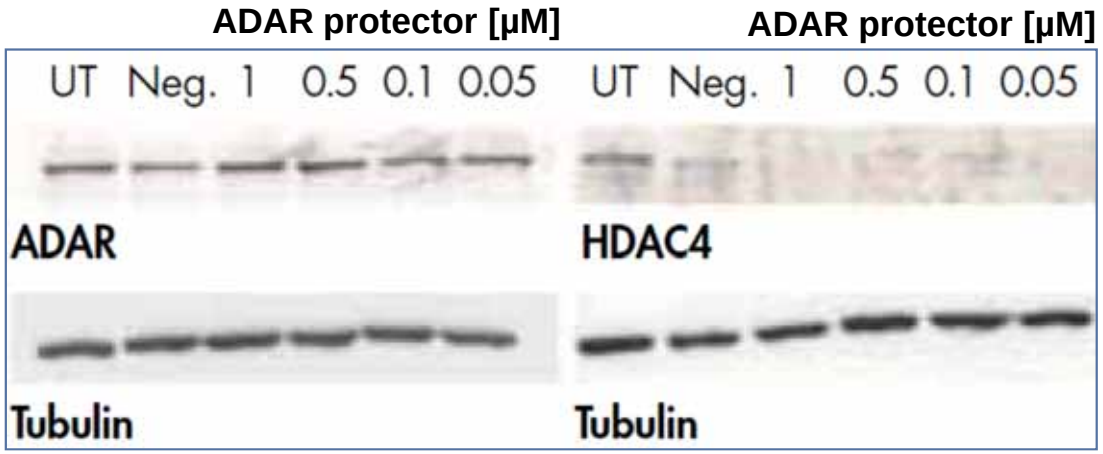
QIAGEN からの推奨 : miScript Target Protector



▶ miScript Target Protector **は、**miRNA **のターゲットを特異的に結合を阻害できる**

Target Protector による特異的阻害例

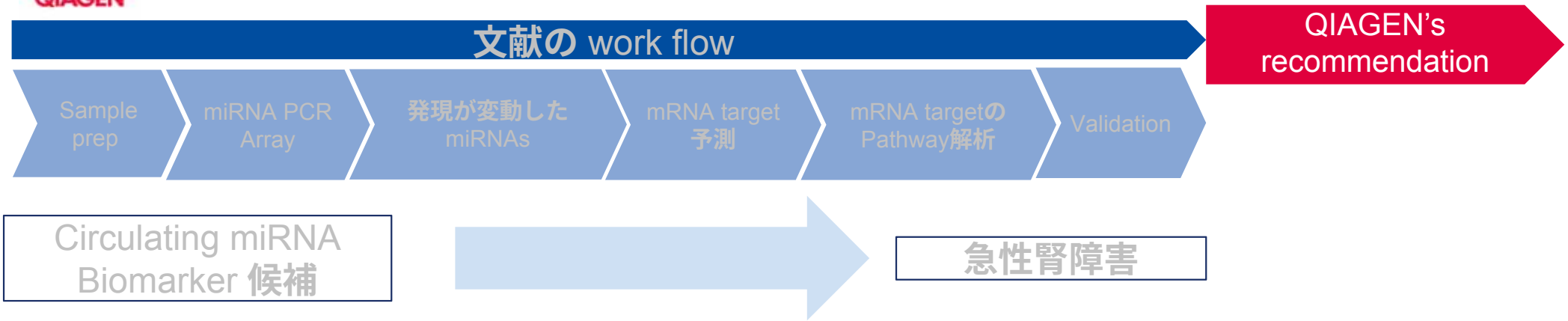




UT（untransfected）
Neg :Negative Control Target Protector

► Target Protector は、miRNAのターゲットを ウエスタンブロット にて簡便に確認可能

文献事例の work flow



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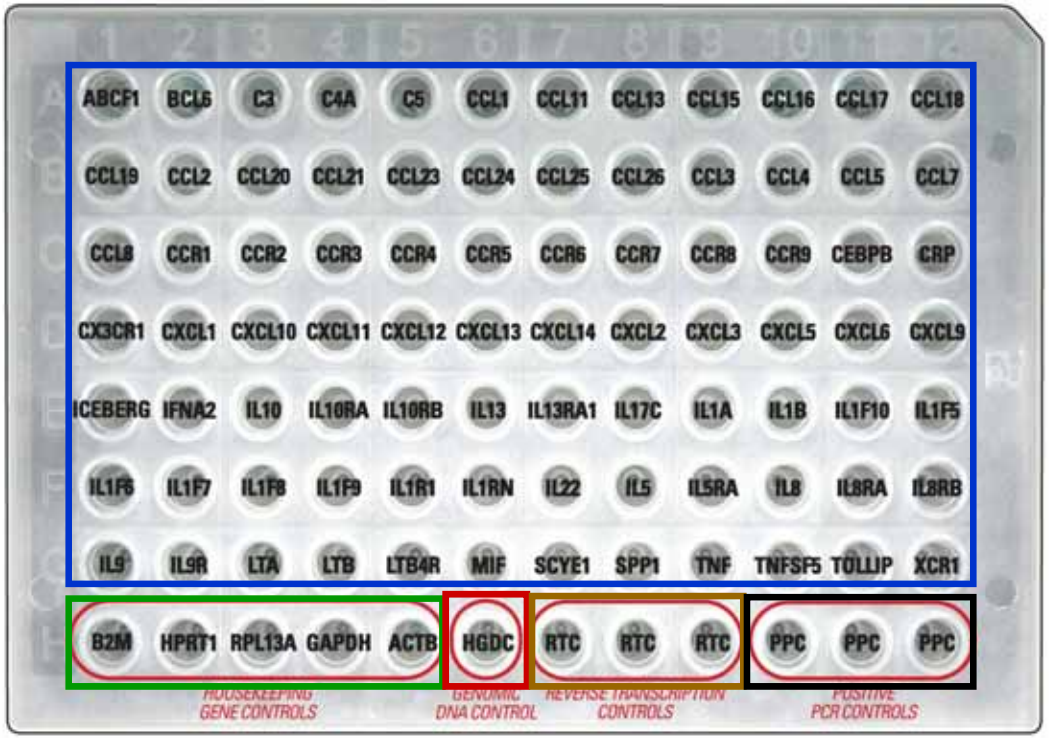
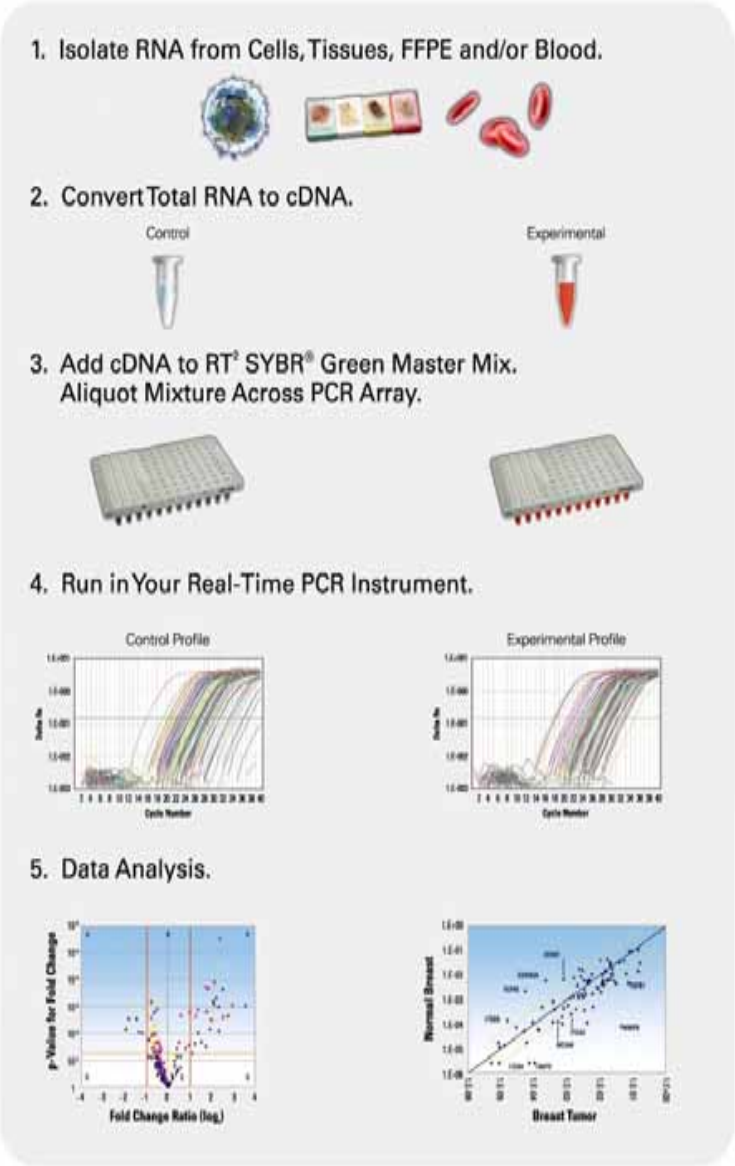
   Nucks1 の下流の遺伝子の発現も解析するべきでは？

QIAGEN からの推奨：RT² Profiler PCR Array

mRNA **に対する**Primer **がスポット済み**
qPCR Plate



RT² PCR Arrays **が搭載している遺伝子は、**
pathway **にフォーカスしている**



約200のパスウェイに対応した、RT2 Profiler PCR Arrays

https://www.qiagen.com/jp/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/rt2-profiler-pcr-arrays/

Cancer and Apoptosis	Cytokines & Inflammation	Development & Stem Cells
Apoptosis	Inflammatory Cytokines	Stem Cells
Cell Cycle	Th17 for Inflammation	WNT Signaling / Notch Signaling
Human miRNA Array (NEW!)	Common Cytokines / Chemokines	Terminal Differentiation Markers
Breast Cancer & Estrogen Receptor	Inflammasomes	TGFβ / BMP Signaling
Tumor Metastasis	NF-kB Signaling Pathway	Endothelial Cell Biology
Epithelial-to-Mesenchymal Transition	Th1-Th2-Th3	Osteogenesis
Angiogenesis	TNF Ligands	Growth Factors
Cancer Drug Resistance	Toll-like Receptors	ECM & Adhesion
Signal Transduction	**Toxicology & Drug Metabolism**	**Neuroscience**
Signal Transduction PathwayFinder	Drug Metabolism	Neuroscience Ion Channels
NFkB Signaling	Drug Phase I Enzymes	Neurotransmitter Receptors
Jak / Stat Signaling	Drug Transporters	Neurotrophins & Receptors
DNA Damage Signaling	Oxidative Stress	Neurogenesis and Neural Stem Cell
Insulin Signaling	Stress & Toxicity	
MAP Kinase Signaling	**Other Diseases**	**Custom PCR Arrays** (H/M/R/Q/D/F)
cAMP / Calcium Signaling	Atherosclerosis	96-Well, 384-Well Plate
p53 Signaling	Diabetes	100-Well Disc, 96x96 Chip

▶ **研究にマッチした PCR Array を選択**

どのArray が研究にマッチしているか？

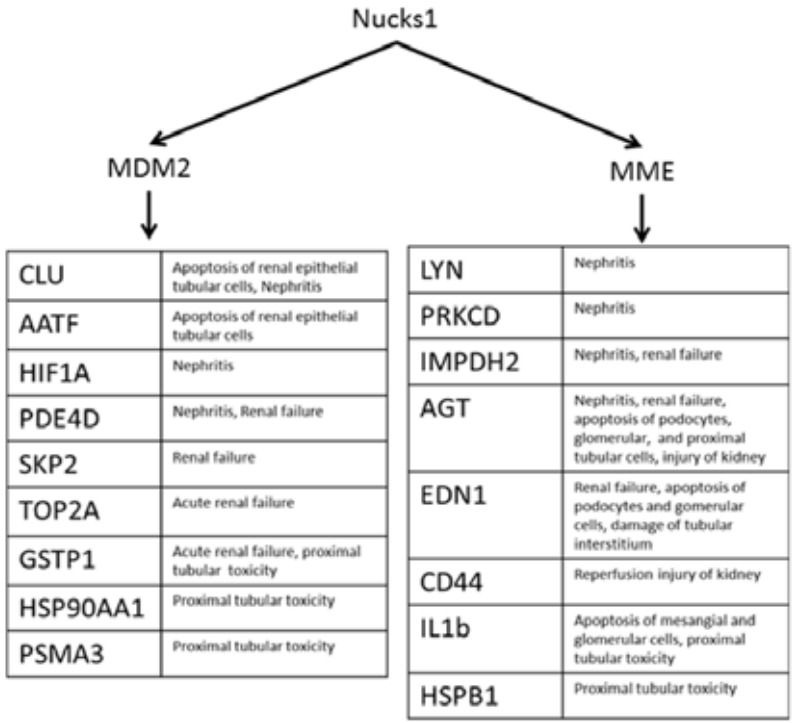


Figure 6. Downstream targets of Nucks1. Pathway analysis was performed on Nucks1 (nuclear substrate 1) using Ingenuity Pathway Analysis. Downstream targets of Nucks1 appear to be involved ir

約200 種類のRT^2 Profiler PCR Arrays

Cancer and Apoptosis	Cytokines & Inflammation	Development & Stem Cells
Apoptosis	*Inflammatory Cytokines*	*Stem Cells*
Cell Cycle	*Th17 for Inflammation*	*WNT Signaling / Notch Signaling*
Human miRNA Array (NEW!)	*Common Cytokines / Chemokines*	*Terminal Differentiation Markers*
Breast Cancer & Estrogen Receptor	*Inflammasomes*	*TGFβ / BMP Signaling*
Tumor Metastasis	*NF-kB Signaling Pathway*	*Endothelial Cell Biology*
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Angiogenesis	*TNF Ligands*	*Growth Factors*
Cancer Drug Resistance	*Toll-like Receptors*	*ECM & Adhesion*
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Signal Transduction PathwayFinder	*Drug Metabolism*	*Neuroscience Ion Channels*
NFkB Signaling	*Drug Phase I Enzymes*	*Neurotransmitter Receptors*
Jak / Stat Signaling	*Drug Transporters*	*Neurotrophins & Receptors*
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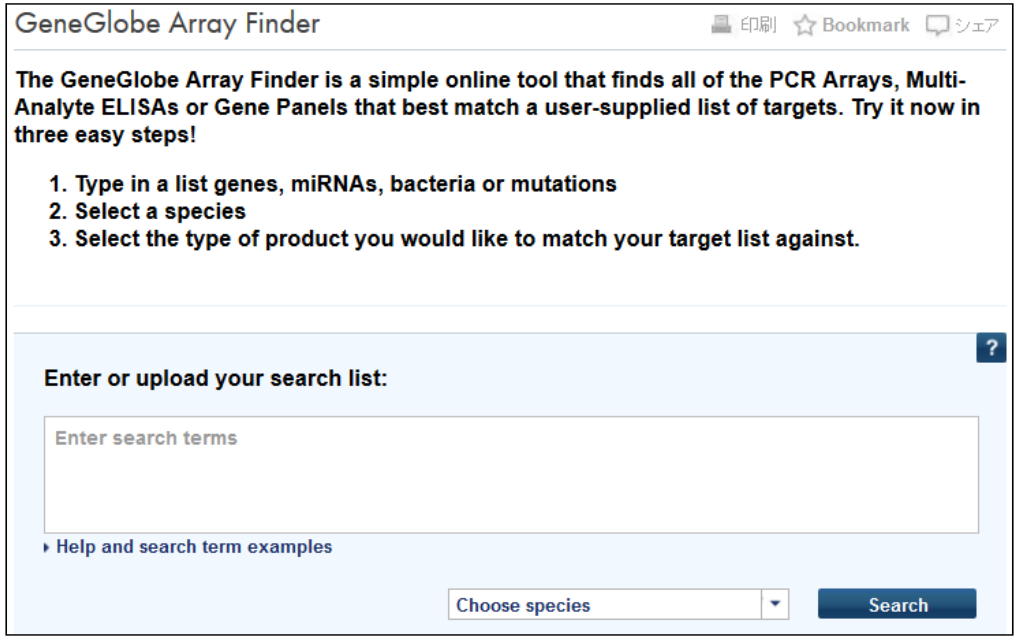
Insulin Signaling	*Stress & Toxicity*	
MAP Kinase Signaling	**Other Diseases**	**Custom PCR Arrays** *(H/M/R/Q/D/F)*
cAMP / Calcium Signaling	*Atherosclerosis*	*96-Well, 384-Well Plate*
p53 Signaling	*Diabetes*	*100-Well Disc, 96x96 Chip*

▶ **どのArray がマッチするかを確認するのは煩雑**

QIAGEN からの推奨 : Array Finder



Array Finder

研究対象の遺伝子を入力

Enter or upload your search list:

mdm2, clu, aatf, hif1a, pde4d, skp2, top2a, gstp1, hsp90aa1, psma3, mme, lyn, prkcd, impdh2, agt, edn1, cd44, il1b, hspb1

Help and search term examples

Choose species
Choose species
Human (Homo sapiens)
Mouse (Mus musculus)
Rat (Rattus norvegicus)
All species
Frog (clawed frog) (Xenopus laevis)
Arabidopsis (Arabidopsis thaliana)
Cow (Bos taurus)

Search

Choose array product type

RT2 Profiler PCR Arrays for mRNA expression
miScript miRNA PCR Arrays for miRNA expression
qBiomarker Somatic Mutation PCR Arrays for cancer mutation detection
qBiomarker Copy Number PCR Arrays for CNV/CNA analysis
EpiTect Methyl qPCR Arrays for DNA methylation
EpiTect ChIP qPCR Arrays for chromatin immunoprecipitation studies

Arrays containing genes of intere

Hit したArray が表示

RT² Profiler™ PCR Array Human Cancer Drug Targets (Cat. No. PAHS-507Z)
6 out of 19 genes show matches — Show Details — configure

RT² Profiler™ PCR Array Human Osmotic Stress (Cat. No. PAHS-151Z)
5 out of 19 genes show matches — Show Details — configure

RT² Profiler™ PCR Array Human Molecular Toxicology PathwayFinder 384HT (Cat. No. PAHS-3401Z)
5 out of 19 genes show matches — Show Details — configure

RT² Profiler™ PCR Array Human Endothelial Cell Biology (Cat. No. PAHS-015Z)
4 out of 19 genes show matches — Show Details — configure

RT² Profiler™ PCR Array Human Cancer Drug Targets (Cat. No. PAHS-507Z)
6 out of 19 genes hide matches

genes match:
MDM2 [Human]
HIF1A [Human]
TOP2A [Human]
GSTP1 [Human]
HSP90AA1 [Human]
PRKCD [Human]

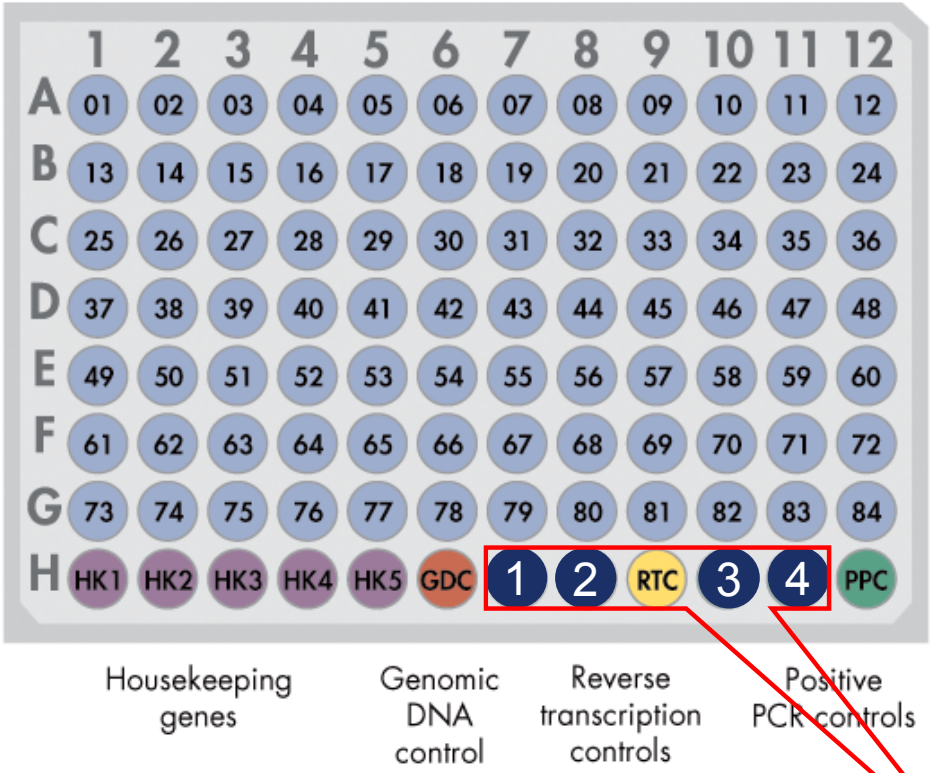
マッチした遺伝子

genes mismatch:
CLU [Human]
AATF [Human]
PDE4D [Human]
SKP2 [Human]
PSMA3 [Human]
MME [Human]
LYN [Human]
IMPDH2 [Human]
AGT [Human]
EDN1 [Human]
CD44 [Human]
IL1B [Human]
HSPB1 [Human]

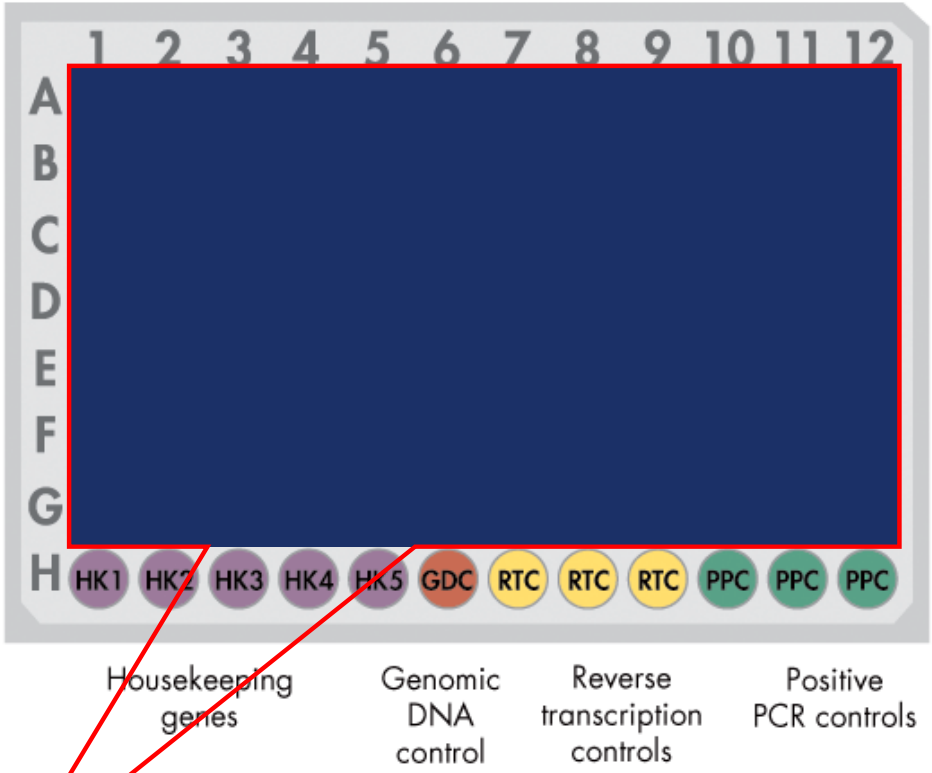
マッチしなかった遺伝子

Modified Array と Custom Array もあります

既存Array をModify



Custom Array



任意の遺伝子を選択出来る

Circulating miRNA バイオマーカー候補と病気の関係を明らかにする

Circulating miRNA Biomarker 候補 → 急性腎障害

文献の work flow → QIAGEN's recommendation

Sample prep → miRNA PCR Array → 発現が変動した miRNAs → mRNA target 予測 → mRNA targetの Pathway解析 → Validation → miRNA Target validation → Validation of the pathway of interest

miRNeasy Serum/Plasma	miScript PCR Array			IPA		Target Protector	RT2 PCR Array

- ✓ Serum/Plasma 用に検証された Protocol がある
- ✓ Spike-In Control により、信頼性のある結果を得られる

- ✓ mRNAの下流を探索し、疾病との関連を見つける事が出来る

- ✓ miRNA Target の確認が、特異的に簡便に出来る

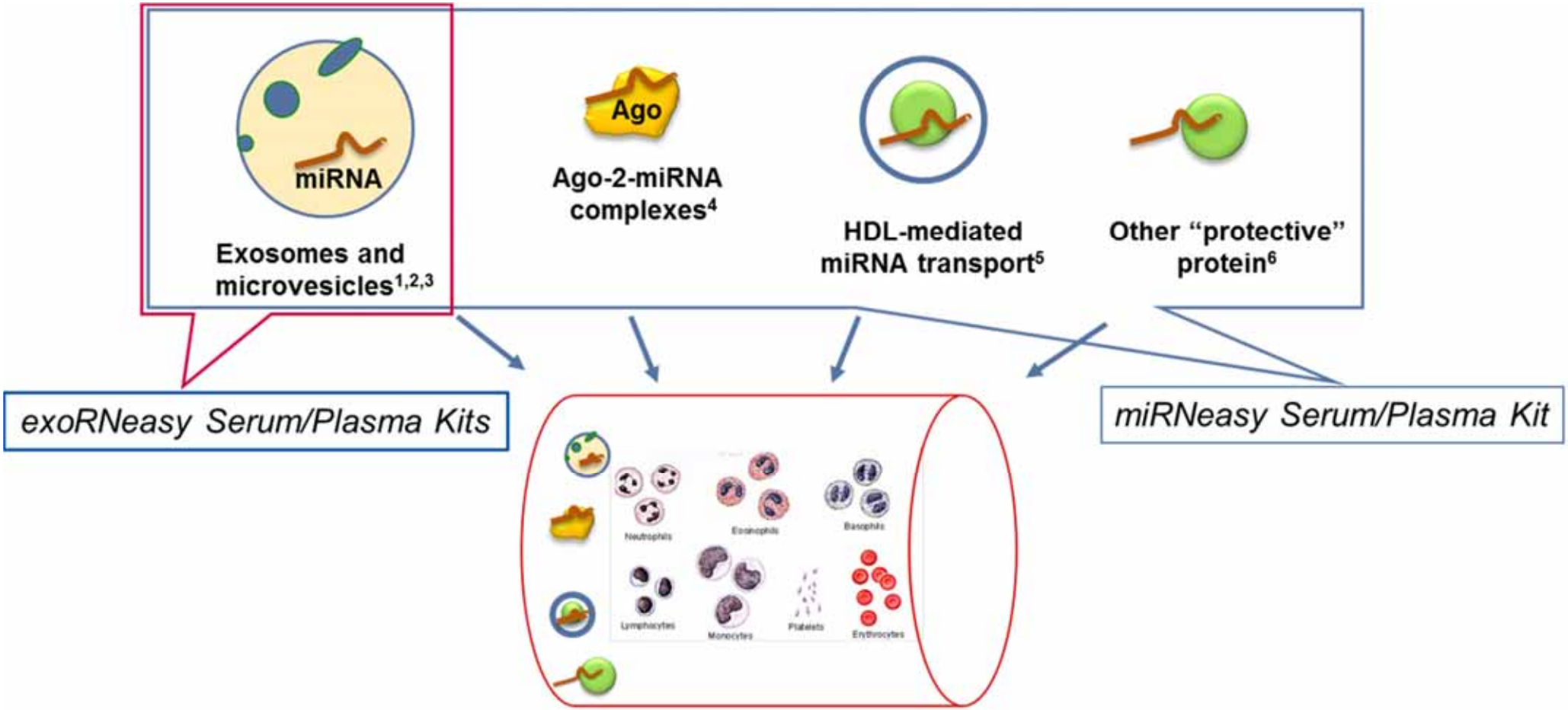
- ✓ Pathway 単位で発現を簡便に検証出来る
- ✓ Array Finder はマッチする Array を簡単に見つける事が出来る

紹介した文献事例

Total のCirculating miRNAを解析

Exosome や細胞外小胞に絞った解析は？

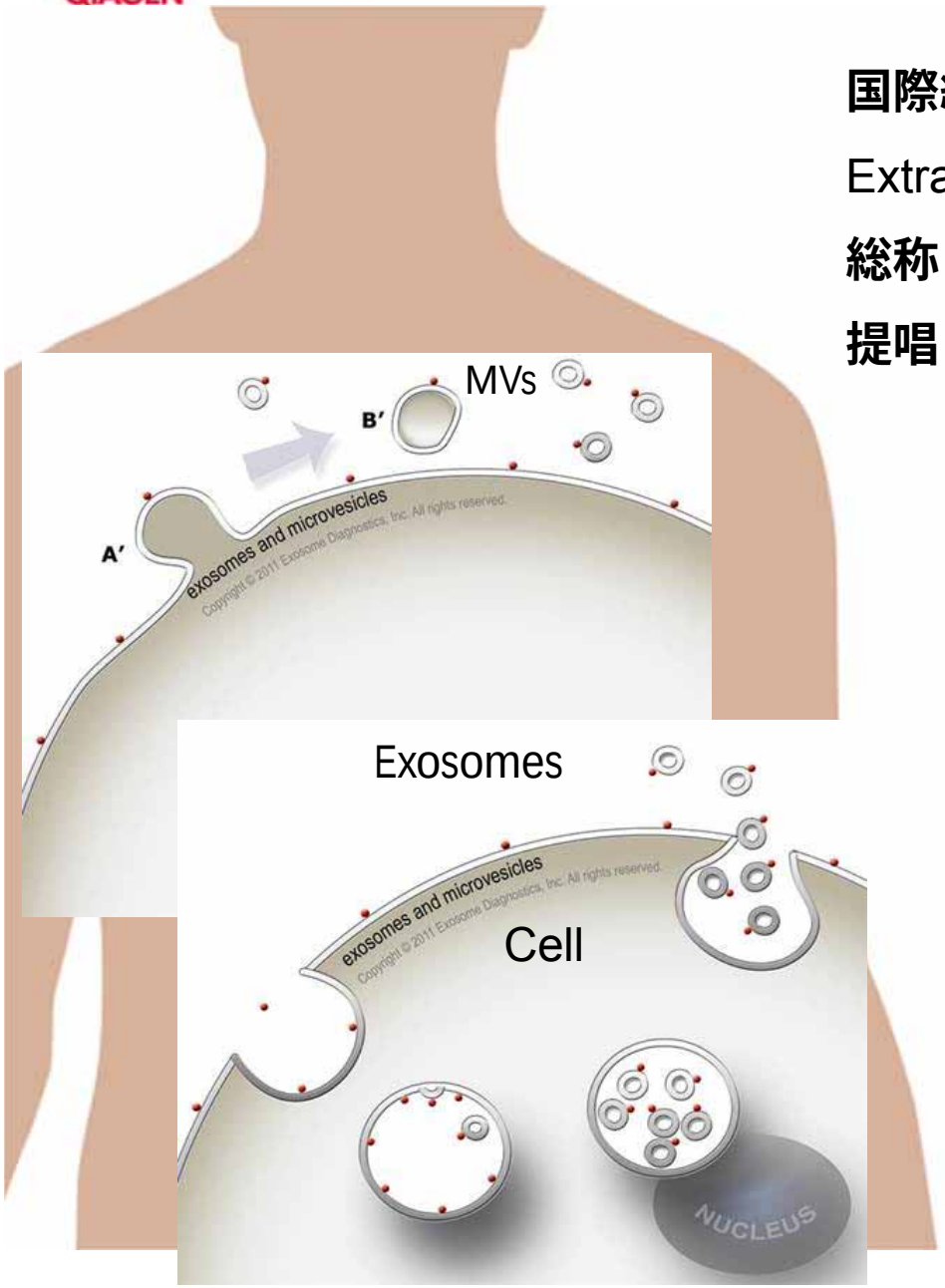
細胞外で安定なmiRNA



miRNA は細胞外で、タンパク質と複合体形成や小胞に内包されており、安定

1) Valadi, H., et.al.,(2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat Cell Biol **9**:654-659
2) Hunter MP et. al., (2008) Detection of microRNA Expression in Human Peripheral Blood Microvesicles, PLoS ONE **3**:e3694
3) Kosaka, N et. al (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells, J Biol Chem **285**: 17442-17452
4) Arroyo, JD et. al., (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc. Natl. Acad. Sci **108**: 5003-5008
5) Vickers, KC., et. al., (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol **13**:423
6) Wang K, Zhang S, Weber J, Baxter D, Galas DJ.(2010) Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res. 2010 Nov 1;**38**(20):7248-59.

Exosomes / Microvesicles



国際細胞外小胞学会（ISEV: International Society for Extracellular Vesicles）**では、これら細胞外分泌顆粒の総称として、**Extracellular Vesicles（EVs）**と呼ぶことを提唱している**

Exosomes、Microvesicles、etc.

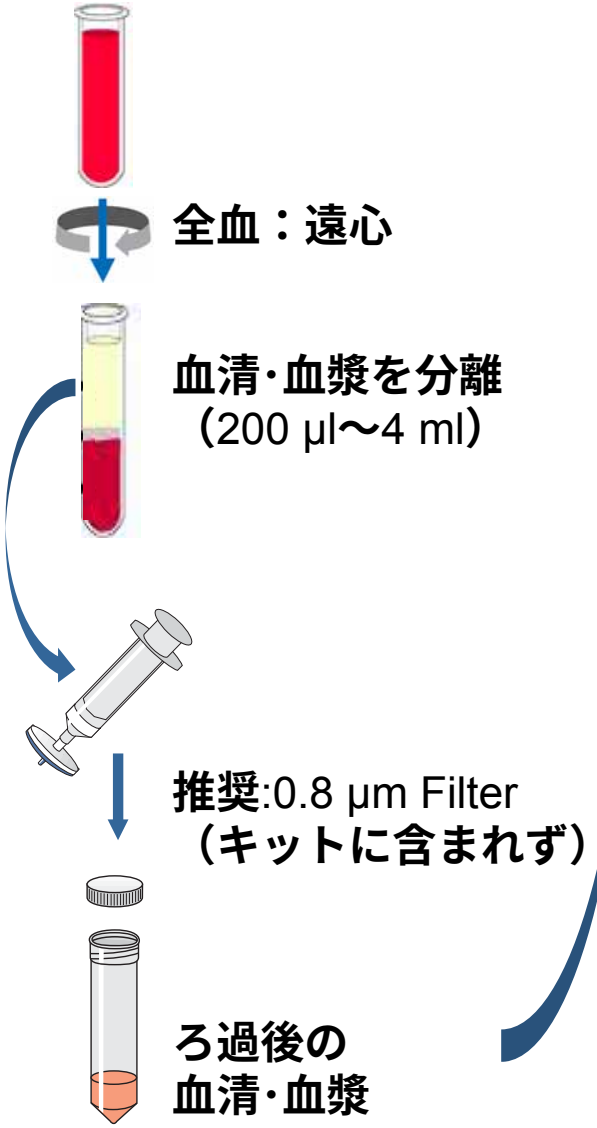
=> 細胞外小胞

Extracellular Vesicles (EVs)

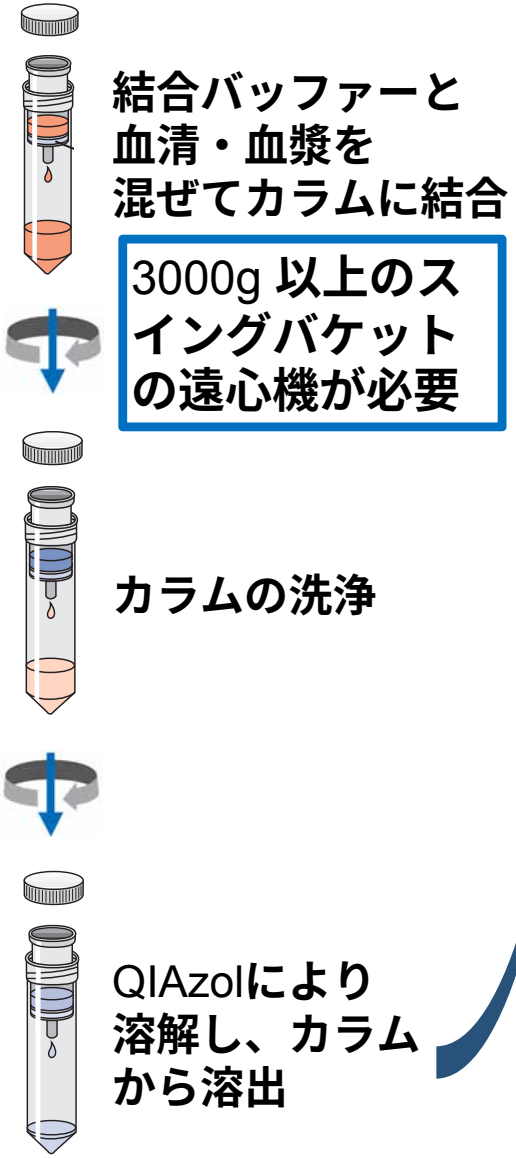
細胞外小胞（Extracellular Vesicles: EVs）特異的RNA 精製キット
exoRNeasy Serum/Plasma Kits

所要時間1時間以内

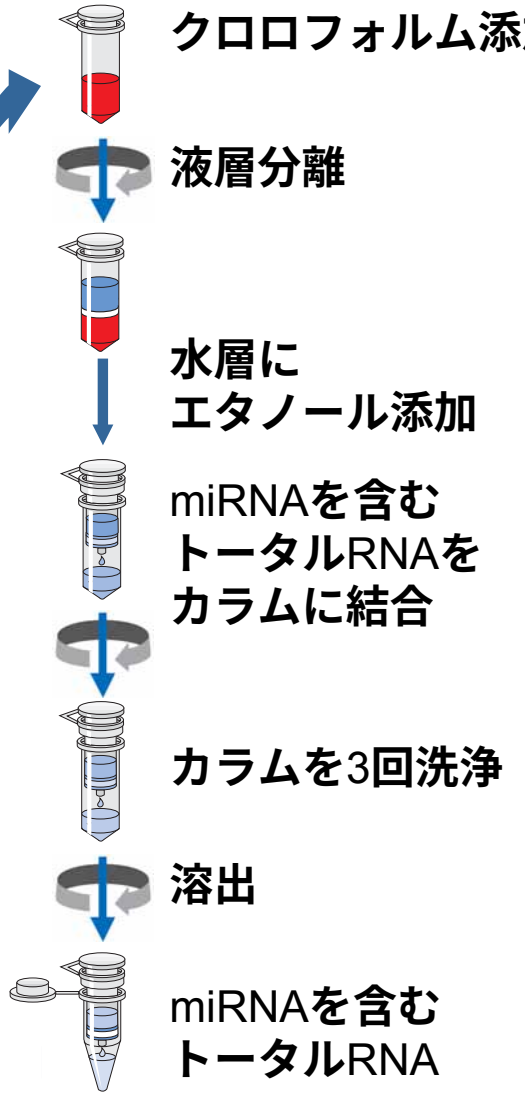
キットはここから



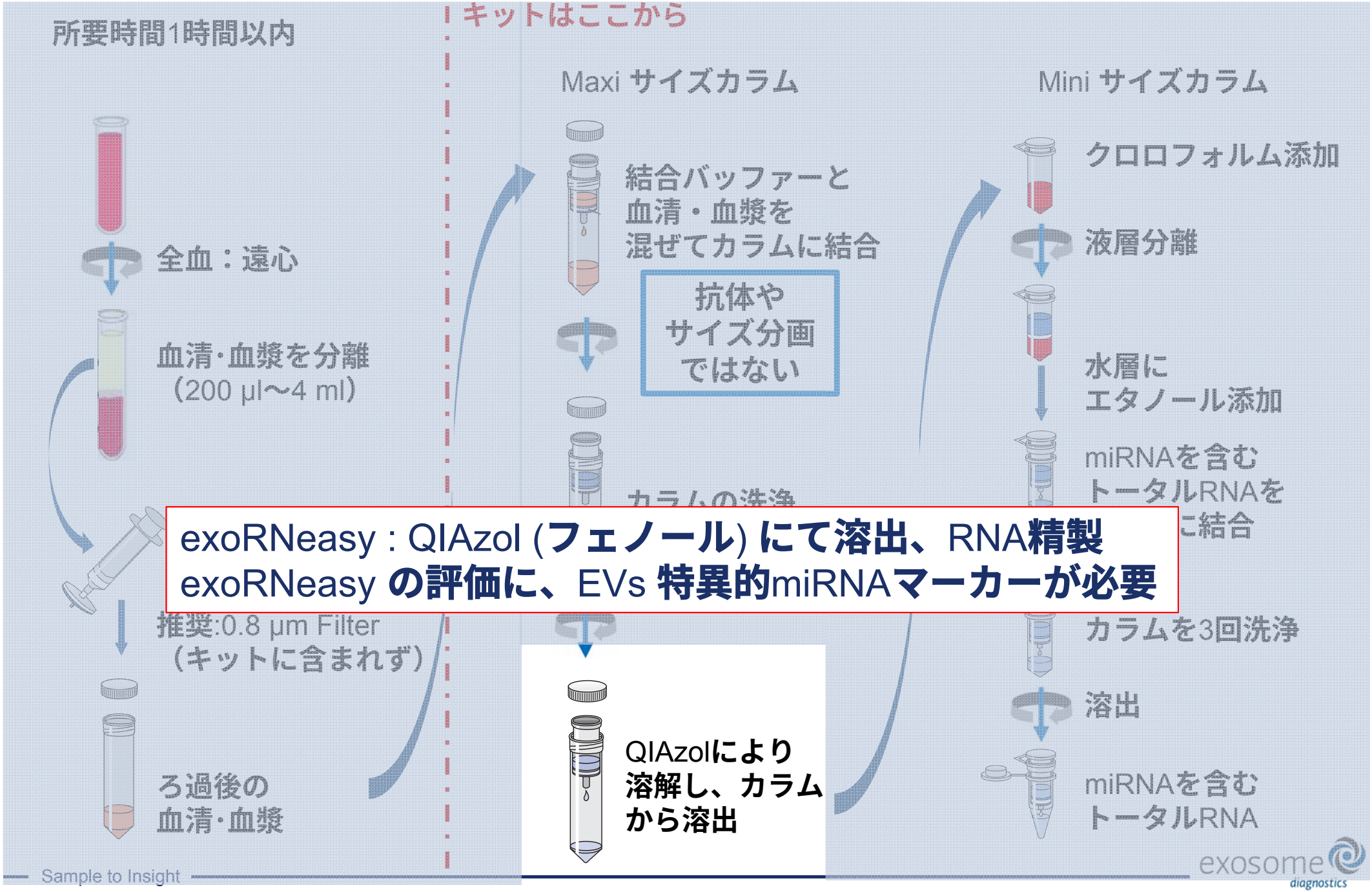
Maxi サイズカラム



Mini サイズカラム



細胞外小胞（Extracellular Vesicles: EVs）特異的RNA 精製キット
exoRNeasy Serum/Plasma Kits

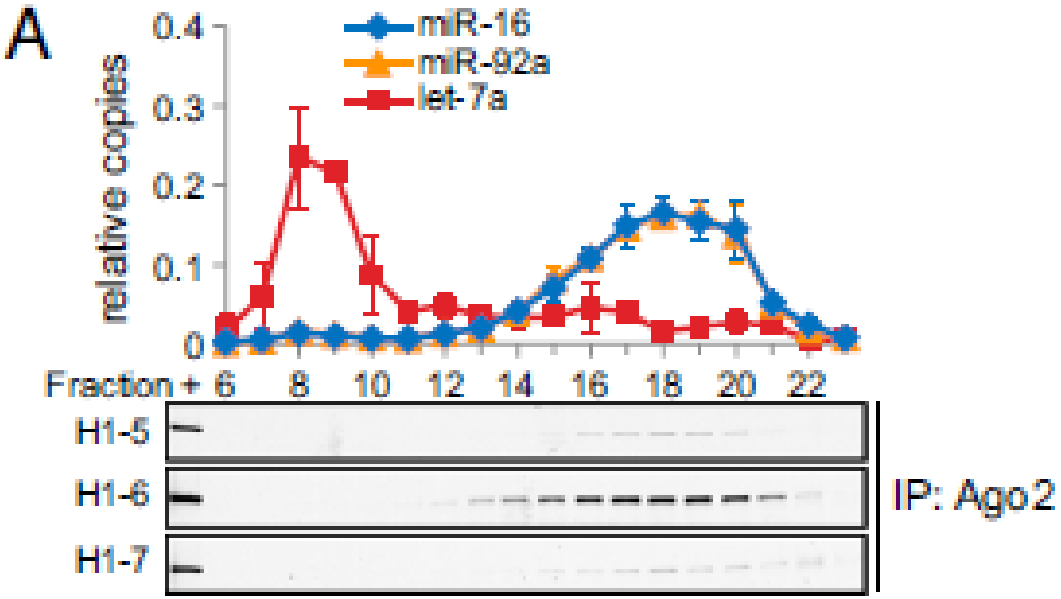


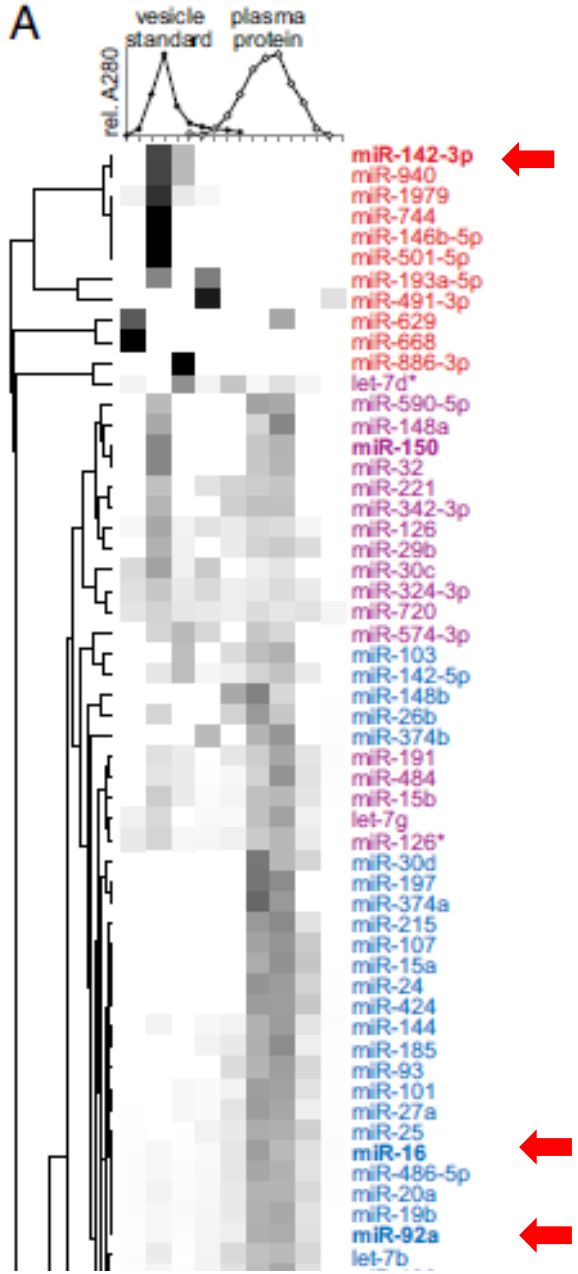
exoRNeasy : QIAzol (**フェノール**) **にて溶出、**RNA**精製**
exoRNeasy **の評価に、**EVs **特異的**miRNA**マーカーが必要**

EVs 特異的miRNAs : QIAGEN が参考にした文献

Arroyo JD et al. Proc Natl Acad Sci USA 2011

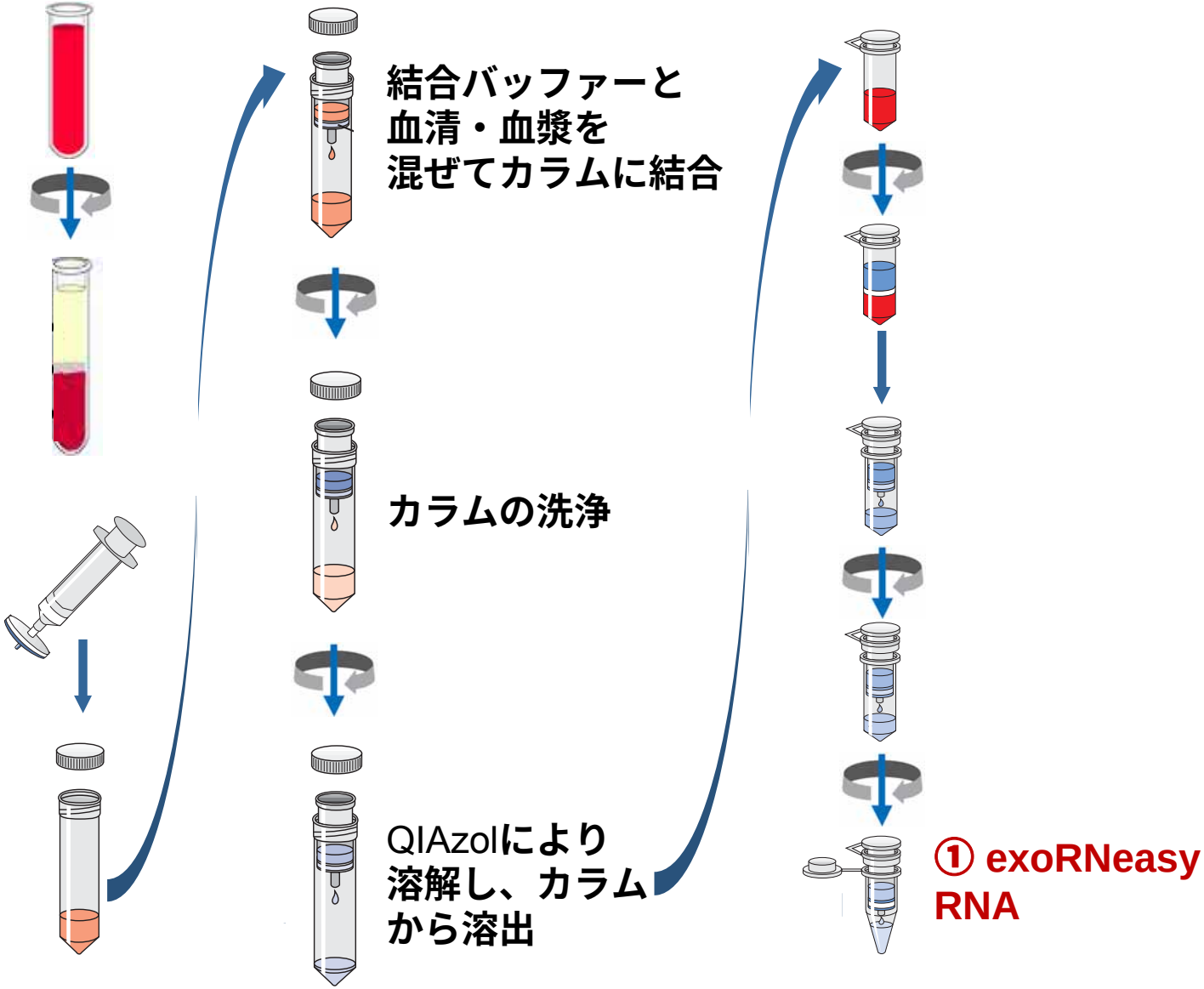
健常人の血漿をゲル濾過、各miRs の局在を解析





- miR-16, miR-92a => Ago2 **分画に局在**
- let-7a, miR-142 => EVs **分画に局在**

exoRNeasy : 特異性の検証



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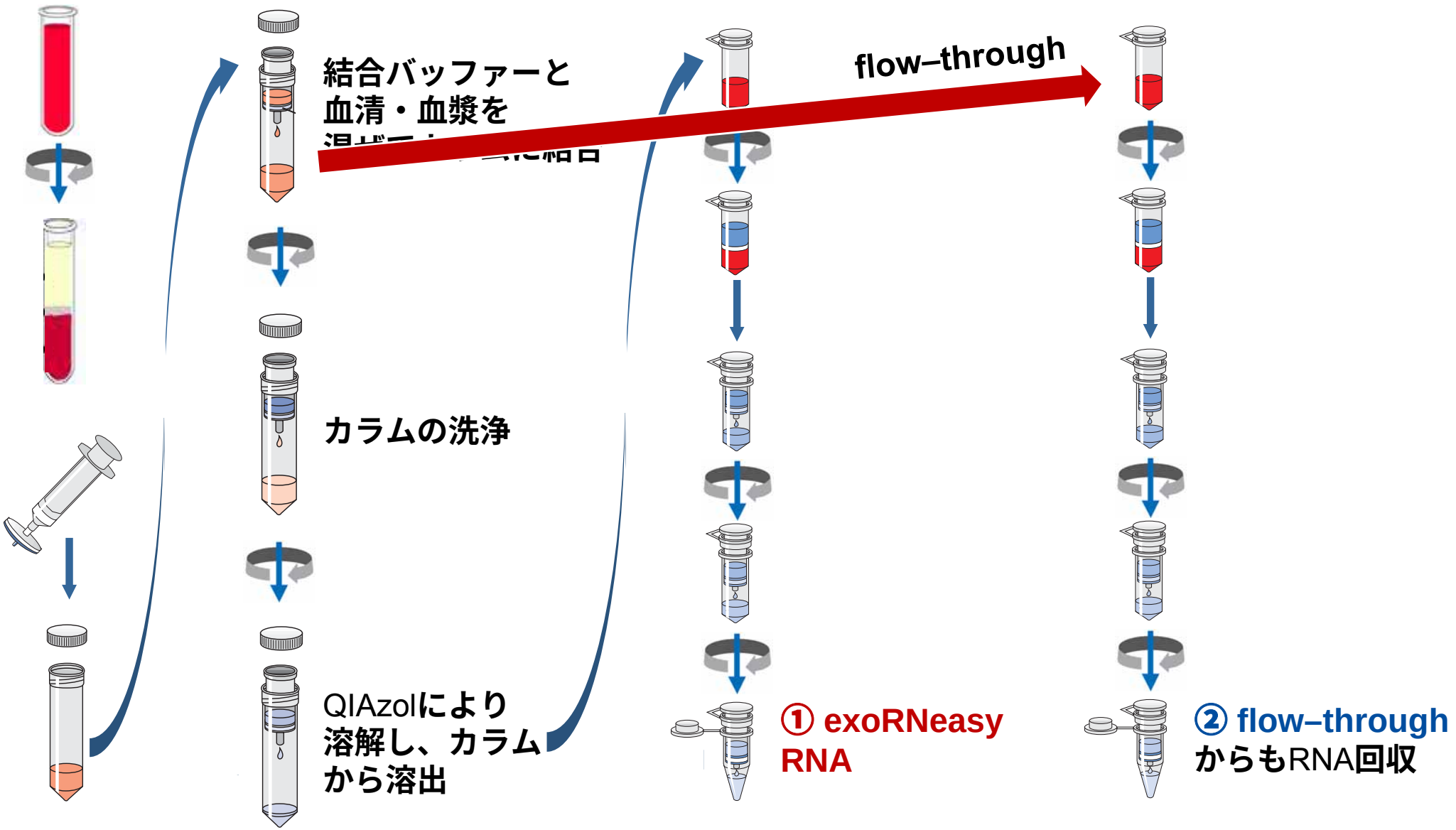
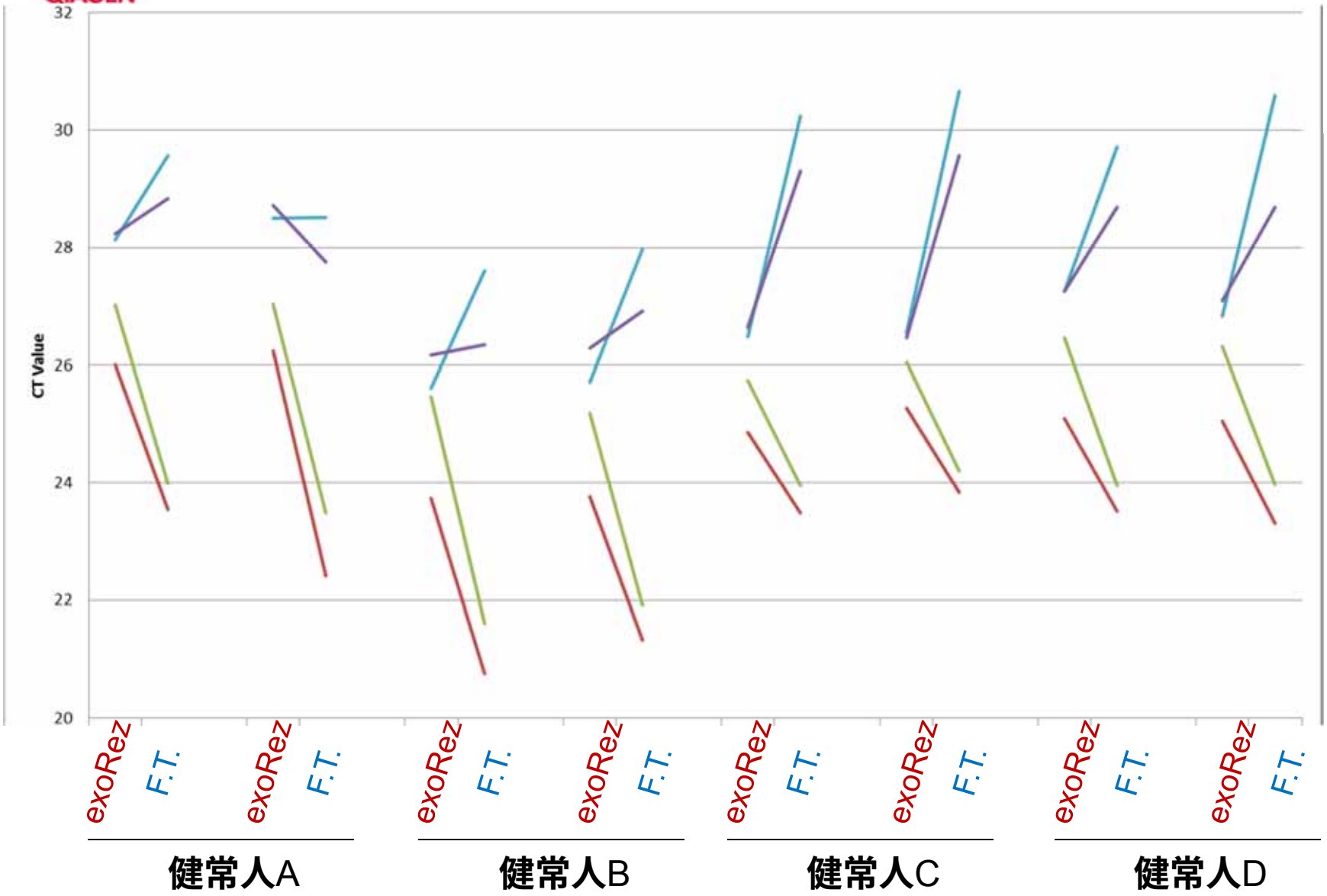


exoRNeasy Kit: 結合したmiRs, 結合しなかったmiRs



Sample
200 µl Serum
健常人

EVs-miRNAs*
- miR-142
- let 7a

Ago2 –miRNAs*
- miR 92a
- miR-16

*Arroyo JD et al. Proc Natl Acad Sci USA 2011

- ▶ exoRNeasy は、EVs 特異的miRs が濃縮された
- ▶ Ago2 特異的 miRs は flow through に排除した

EVs 特異的 miRNA バイオマーカー候補と病気の関係を明らかにする

EVs 特異的miRNA マーカー候補 → 疾病 Phenotype

QIAGEN's recommendation work flow

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IPA

- ✓ mRNAの下流を探索し、病気との関連を見つける事が出来る

Target Protector

- ✓ miRNA Target の確認が、特異的に簡便に出来る

RT2 PCR Array

- ✓ Pathway 単位で発現を簡便に検証出来る
- ✓ Array Finder はマッチする Array を簡単に見つける事が出来る

QIAGEN New Products

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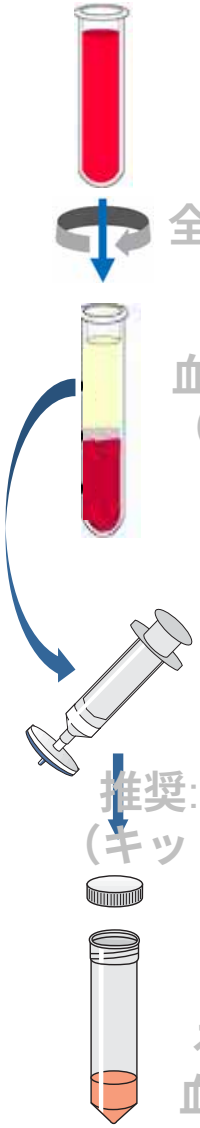
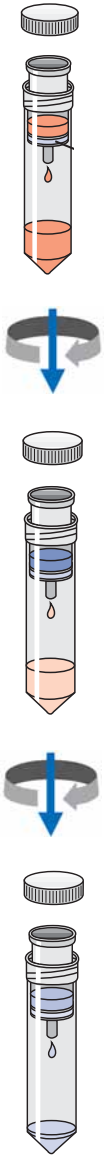
exoRNeasy Serum/Plasma Kits

所要時間1時間以内

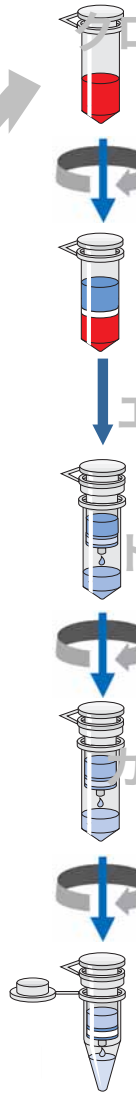
キットはここから



Maxi サイズカラム

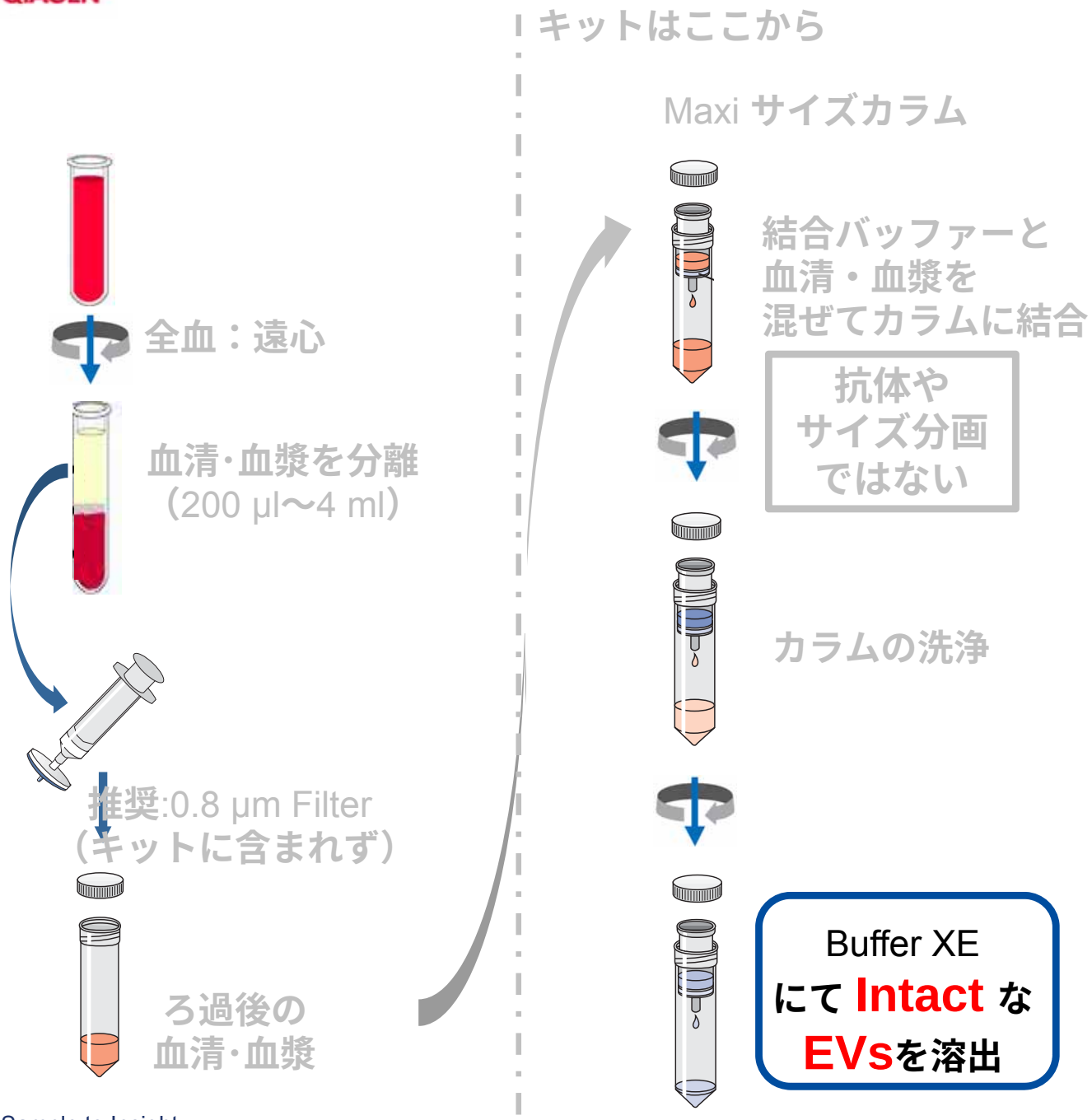


Mini サイズカラム



Intact な EVs の精製: exoEasy Maxi Kit

近日発売



所要時間約25分

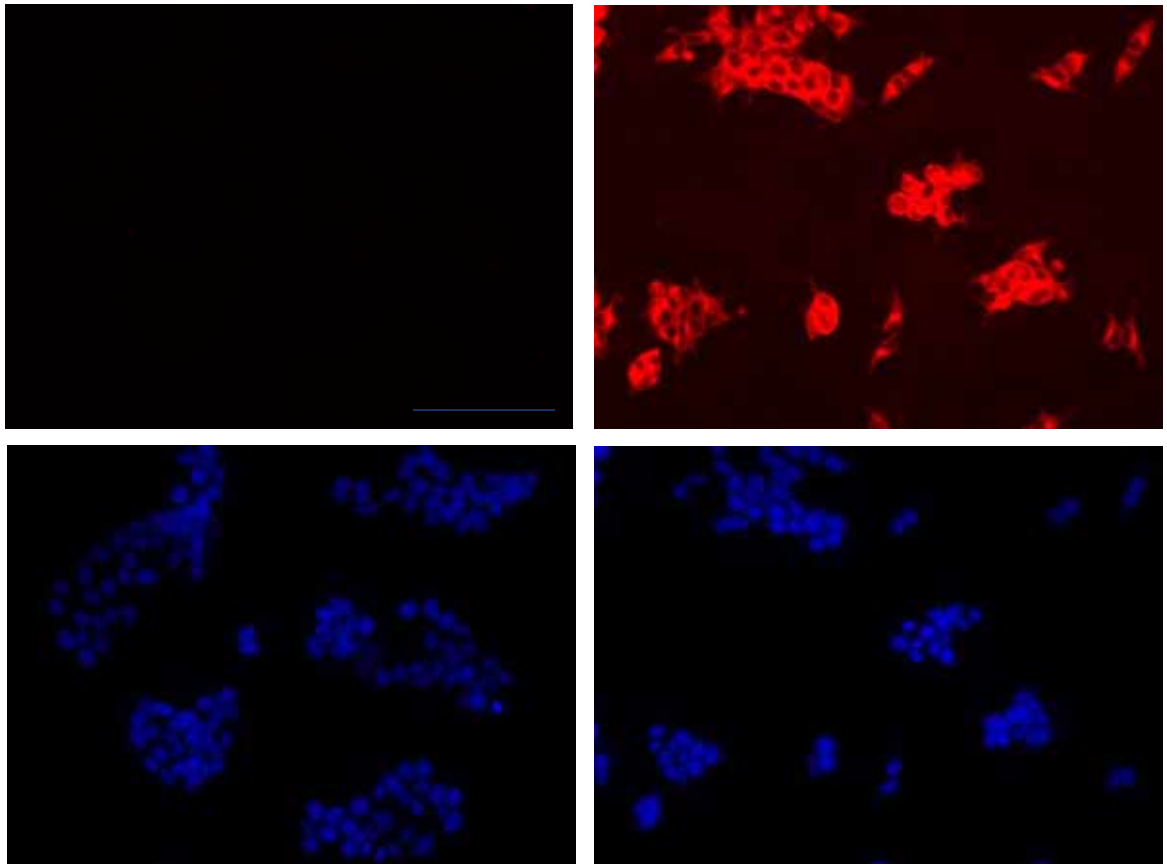
exoEasy Kit : 精製したEVs の細胞への取り込み

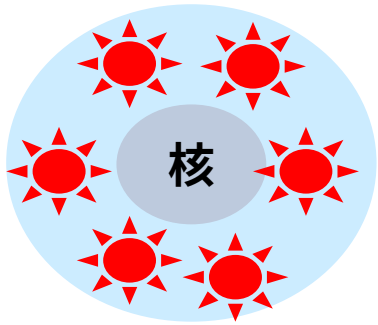
- HEK 293T cells で産生されたEVs をexoEasy にて精製し、限外ろ過にてbuffer交換。Bodipy-ceramide を用いて標識
- 293T cells への取り込み: 1.45E9 particles (Nanosight により測定) を $2x10^4$ cells にアプライし1時間インキュベーション

Dye のみ (neg. control)　　Dye + exoEasy によるEVs

Bodipy (EVs)

DAPI (Nuclei)





⇒ Bodipy 標識 EVs によって細胞質が染まり、細胞内に取り込まれた

miRNA バイオマーカー候補と疾病の関係を結ぶ work flow

Circulating miRNA Biomarker 候補 → 疾病 Phenotype

QIAGEN's recommendation work flow

Sample prep	miRNA PCR Array	発現が変動した miRNAs	mRNA target 予測	mRNA targetの Pathway解析	miRNA Target validation	Validation of the pathway of interest
Total の Serum/Plasma miRNeasy Serum/Plasma 又は EVs 特異的 RNA exoRNeasy Serum/Plasma	miScript PCR Array			IPA	Target Protector	RT2 PCR Array