RESULTADO DO JULGAMENTO DO RECURSO CONTRA RESULTADO O DA PROVA ESCRITA DO CONCURSO PÚBLICO DE PROVAS E TÍTULOS PARA PROFESSOR EFETIVO TITULAR-LIVRE DA CARREIRA DO MAGISTÉRIO SUPERIOR DA ESCOLA DE VETERINÁRIA E ZOOTECNIA DA UNIVERSIDADE FEDERAL DE GOIÁS (EVZ/UFG), NA ÁREA DE BIOLOGIA CELULAR E MOLECULAR APLICADAS À CIÊNCIA ANIMAL, IMPETRADO PELO CANDIDATO RINALDO WELLERSON PEREIRA.

A comissão de julgamento de recurso contra o resultado da prova escrita, de caráter eliminatório, do concurso público de provas e títulos para professor efetivo titular-livre da carreira do magistério superior, da Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, na área de Biologia Celular e Molecular Aplicadas à Ciência Animal, nomeada pela portaria EVZ/2015 nº 12, de 12/05/2015, e constituída pelos professores doutores Naida Cristina Borges (Coordenadora do PPGCA) - Presidente; Veridiana Maria Brianezi Dignani de Moura (Chefe do Departamento de Medicina Veterinária) - Membro Titular; Rosângela de Oliveira Alves Carvalho (Coordenadora do Curso de Medicina Veterinária) - Membro Titular, e Paulo Henrique Jorge da Cunha (Diretor do HV) - Membro Suplente, recebeu da direção da EVZ, no dia 27/05/2015, às 15h30min, o recurso apresentado pelo candidato RINALDO WELLERSON PEREIRA contra o resultado da sua prova escrita.

Inicialmente, a comissão recursal solicitou à direção da EVZ/UFG a prova escrita do candidato, as atas do concurso e toda documentação pertinente (edital, normas complementares, resoluções, etc.). De posse dessa documentação, a comissão avaliou o conteúdo apresentado no recurso impetrado pelo candidato, tendo como base a legislação que rege o concurso (Resolução Conjunta CONSUNI/CEPEC Nº 02/2013).

O candidato manifestou que a sua eliminação foi baseada em uma "chave de resposta", termo inexistente nas atas exaradas pela Banca Examinadora. Cabe ressaltar que os critérios de correção utilizados pela Banca Examinadora constam na ata de correção da prova escrita e contemplaram os seguintes itens: "*1) Introdução (apresentação do tema RNA, composição e estrutura, tipos de RNA, função na célula); 2) Cuidados na coleta das amostras visando a extração de RNA; 3) Extração de RNA em amostras de tecidos e células; 4) Quantificação de RNA; 5) Qualidade do RNA (Degradação e Contaminação); 6) Armazenamento; 7) Pontos Críticos na utilização de RNA; 8) Bibliografia indicada.*" À luz desses itens, a Banca Examinadora avaliou a prova escrita do candidato, sendo que os membros atribuíram individualmente a nota 6,0 (seis vírgula zero), refletindo média 6,0 (seis vírgula zero), o que, de acordo com as normas complementares do concurso - item III, alínea "a" - torna o candidato não apto a continuar no concurso por não ter atingido a nota mínima de 7,0 (sete vírgula zero) pontos.

Em seu documento de recurso, o candidato afirma "*O conhecimento demonstrado na dissertação em linguagem acadêmica reflete a experiência que eu tenho, orientando alunos em nível de Pós Graduação e em artigos publicados em revistas com revisão por pares e indexadas*". Acerca dessa afirmação, cabe ressaltar que nos documentos oficiais a banca examinadora não avaliou o conhecimento global, a produção científica e a experiência do candidato, tendo avaliado apenas o documento produzido pelo candidato no momento da realização de sua prova escrita, julgando o mesmo insuficiente.

A manifestação do candidato desqualificando a banca examinadora, em virtude de não encontrar nos currículos dos mesmos, na plataforma Lattes do CNPq, uma única palavra-chave mRNA não se

sustenta sob o ponto de vista da legislação que rege o concurso, uma vez que o candidato teve a oportunidade de apresentar recurso contra qualquer membro da banca assim que a mesma foi publicada no sistema de concurso da UFG, na data de 12/05/2015. O parágrafo primeiro do Artigo 12 da Resolução Conjunta CONSUNI/CEPEC Nº 02/2013 especifica *"que qualquer candidato com inscrição homologada poderá alegar suspeição contra qualquer membro ou suplente da Banca Examinadora, para o Conselho Diretor da Unidade Acadêmica interessada no concurso, no prazo de dois dias úteis, a contar da publicação da banca. "* Reforça-se que a banca foi constituída por cinco renomados e conceituados professores titulares de cinco diferentes Universidades da mais alta qualidade (UFG, PUC-GO, UFMG, UNESP-Jaboticabal e UNESP-Botucatu). É oportuno ressaltar que o candidato somente questionou a competência dos membros da Banca Examinadora após tomar ciência de sua eliminação da prova escrita.

O candidato alega que "*Durante a sessão pública de divulgação do resultado, eu não fui informado sobre minha nota e tampouco sobre a chave de resposta utilizada para correção de minha prova. "* Embora não houvesse impedimento legal quanto à divulgação da nota, a Banca Examinadora optou por expressar o resultado da prova escrita anunciando a eliminação do candidato, sendo a sua nota registrada na ata da correção da prova escrita, o que não fere o Artigo 15 da Resolução Conjunta CONSUNI/CEPEC Nº 02/2013, que determina: "*No caso em que a prova for eliminatória e obtido o resultado da prova escrita ou Teórico-prática, a Banca Examinadora proclamará, em sessão pública, o seu resultado, redigindo ata da qual constarão os nomes dos candidatos aprovados na ordem decrescente de classificação*."

Em relação às solicitações enumeradas pelo candidato em seu documento de recurso, a comissão recursal expressa o que segue.

Item 1. O encaminhamento deste recurso para a Comissão previamente constituída, com pelo menos três membros, previamente indicados pelo Conselho Diretor da Unidade para que conheça e decida sobre o presente recurso no prazo regimental, previsto no parágrafo único do artigo 16 da Resolução Conjunta/Consuni/CEPEC n 02/2013.

Item contemplado neste documento.

Item 2. Que a Banca Examinadora dê publicidade à "chave de resposta" exigida ao ponto sorteado pelo candidato RINALDO WELLERSON PEREIRA, mesmo que na Resolução Conjunta - Consuni/CEPEC n 02/2013, bem como nas Normas Complementares ao Edital 73/2014, não vinculem expressamente a resposta da prova escrita a um padrão de "chave de resposta";

A solicitação do candidato não encontra respaldo na legislação que rege o concurso. Conforme anteriormente citado neste documento, a Banca Examinadora registrou em ata os critérios de correção.

Item 3. Que a Banca Examinadora, em sessão pública, informe ao candidato RINALDO WELLERSON PEREIRA sua nota na prova escrita;

Tal solicitação não encontra respaldo na legislação que rege o concurso em questão, conforme citado anteriormente. Reitera-se que a nota do candidato consta da ata de correção da prova escrita.

Item 4. Que seja dada publicidade ao espelho de resposta da Prova Escrita do Candidato pela página SISCONCURSOs da UFG;

A solicitação do candidato não é prevista na resolução que rege o concurso de que trata este documento. Reitera-se que os critérios de correção da prova escrita adotados pela banca examinadora constam deste documento e da ata de correção da prova escrita.

Item 5. Seja dado provimento ao presente recurso determinando que a prova escrita do candidato RINALDO WELLERSON PEREIRA seja avaliada conforme estabelecido no artigo 20 da Resolução Conjunta/Consuni/CEPEC n 02/2013, bem como nas Normas Complementares ao Edital 73 /2014;

A comissão recursal entende que a prova escrita do candidato foi avaliada pela banca examinadora de forma adequada, obedecendo a Resolução Conjunta CONSUNI/CEPEC Nº 02/2013 e as Normas Complementares do Concurso.

Item 6. A reintegração do candidato RINALDO WELLERSON PEREIRA ao concurso público para as etapas de Prova Oral na forma de Conferência Pública e para a Defesa do Memorial.

A comissão recursal nega provimento à retomada das atividades do concurso por entender que a Banca Examinadora avaliou corretamente o candidato, considerando tratar-se de um concurso para Professor Titular-Livre, que representa o topo da carreira acadêmica, eliminando o candidato à luz a legislação que rege o concurso.

Item 7. Que seja dada publicidade a este recurso.

Em consulta ao Diretor da Escola de Veterinária e Zootecnia da UFG, Prof. Dr. Marcos Barcellos Café, a comissão recursal foi informada de que o recurso apresentado pelo candidato será publicado, na íntegra, em anexo, a este documento, no sítio eletrônico http://sistemas.ufg.br/CONCURSOS_WEB/.

Diante do exposto e fundamentada na legislação pertinente, a comissão recursal conclui pelo INDEFERIMENTO da solicitação do candidato.

Goiânia, 28 de maio de 2015.

Prof.ª Dr.ª Naida Cristina Borges
(COORDENADORA DO PPGCA)
PRESIDENTE

Prof.ª Dr.ª Veridiana Maria Brianezi Dignani de Moura
(CHEFE DO DEPARTAMENTO DE MEDICINA VETERINÁRIA)
MEMBRO TITULAR

Prof.ª Dr.ª Rosângela de Oliveira Alves Carvalho
(COORDENADORA DO CURSO DE MEDICINA VETERINÁRIA)
MEMBRO TITULAR

Ao Prof. Dr. Marcos Barcellos Café
Diretor da Escola de Veterinária e Zootecnia da UFG

Eu, Rinaldo Wellerson Pereira, inscrito no Concurso Público para a área de concentração Biologia Celular e Molecular Aplicadas à Ciência Animal, estabelecido através do EDITAL Nº. 73/2014 – Publicado no DOU em 21/11/2014, realizado para Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, para provimento na Carreira de Magistério Superior – Professor Titular Livre do Quadro de Pessoal da Universidade Federal de Goiás, valendo-me da prerrogativa que me é assegurada pelo disposto no artigo 16 da Resolução Conjunta – CONSUNI/CEPEC Nº 02/2013, venho, respeitosamente, pelas razões abaixo expostas, apresentar:

RECURSO CONTRA RESULTADO, DE CARÁTER ELIMINATÓRIO, DA PROVA ESCRITA DO SUPRACITADO CONCURSO PÚBLICO

Aos 25 dias do mês de Maio, eu me apresentei para a instalação do Concurso acima referido, na Sala de Reuniões do Conselho Diretor da Escola de Veterinária. Em seguida à tomada dos documentos, foi sorteado o ponto para a prova escrita.

O ponto sorteado para a prova escrita foi o de número 2, que de acordo com as normas complementares ao Edital Nº 73/2014 tinha como título **"Identificação de RNA em amostras de tecidos e células"**.

Às oito horas do dia 26 de Maio de 2015 eu me apresentei para a prova escrita. Durante 2 horas e 40 minutos dissertei sobre o tema sorteado de maneira manuscrita em folhas de papel almaço como previsto no **Item III**, **letra e** das Normas Complementares ao Edital N 73/2014.

Diz o artigo 20, caput, da Resolução Conjunta - CONSUNI/CEPEC Nº 02/2013 que regulamenta o ingresso para a Carreira de Magistério Superior e para o Cargo Isolado de Professor Titular-Livre do Magistério Superior na Universidade Federal de Goiás:

> *"Artigo 20. A prova escrita terá como objetivo avaliar os conhecimentos do candidato, assim como a sua capacidade de expressão em linguagem acadêmica"*

Fundamentado no referido artigo e na dissertação apresentada à banca, eu discordo de minha eliminação do concurso baseada na afirmação feita pelo presidente da Banca, em sessão pública, que a minha eliminação ocorreu em função da dissertação apresentada **não estar de acordo com a "chave de resposta"**.

Ressalto que em **NENHUM** ponto da Resolução Conjunta - Consuni/CEPEC N 02/2013, bem como em **NENHUM** ponto das Normas Complementares ao Edital 73/2014, está dito que a prova escrita no modelo discursivo deveria atender a **alguma limitação de abordagem referente ao tema.**

Cumpre salientar ainda que o resultado, ao ser proclamado em sessão pública, de sua referida ata cuja cópia acompanha o presente recurso, consta somente que eu fui ELIMINADO. Ao utilizar uma "chave de resposta", seria razoável que esta seja publicada juntamente com o resultado, e que a esta seja dada publicidade, anunciando-a na sessão pública pra divulgação do resultado. Sem esta publicação e informação, o direito constitucionalmente reconhecido a qualquer cidadão ao contraditório e à ampla defesa fica severamente comprometido.

Assim, conforme o ítem III, alínea "c" das Normas Complementares ao EDITAL Nº. 73/2014 o ponto sorteado para a prova escrita foi **"Identificação de RNA em amostras de tecidos e células".** Tema este, como todos os outros, exaustivamente preparado e planejado com antecedência à instalação do concurso. O planejamento da dissertação de cada um dos temas foi compartilhado com acadêmicos com ampla experiência em Biologia Celular e Molecular e todos manifestaram acordo com a abordagem planejada em caso de sorteio de um dos temas. E foi deste processo de preparação que eu planejei e executei minha prova escrita, em forma discursiva, no dia 26 de março de 2015, com a seguinte estrutura, que pode ser constatada através de minha prova escrita:

Introdução

Dissertei sobre a estrutura do RNA e sobre a diversidade de famílias e suas funções na fisiologia celular. Fechei a introdução chamando a atenção para os mRNAs (RNAs mensageiros) e em para os microRNAs. Assim o fiz, pois são estes as principais espécies de RNA identificados em tecidos e células. O que pode ser facilmente comprovado por uma busca em base de referências bibliográficas.

O racional de minha dissertação foi o de dividir a identificação de RNA em tecidos e células em dois tópicos distintos. O primeiro, tratou da utilização de métodos que identificam o RNA em tecido e células mas não mantêm o contexto espacial na célula ou tecido. Já o segundo tratava dos métodos que identificam o RNA e permite a contextualização no ambiente celular.

No tópico Identificação de RNA em tecidos e células sem a manutenção do contexto espacial foi feita a seguinte divisão de subtópicos:

- Extração de RNA de tecidos e células ressaltando a importância de cuidados com RNAse.
- Quantificação de RNA por Absorvância em luz UV e por métodos fluorimétricos
- Controle de qualidade do RNA medindo a contaminação por proteínas utilizando a razão de Absorvância 260/280nm. E o método de eletroforese em chip para medir a integridade (RIN).

Então, dissertei sobre métodos para identificação individual de RNAs em tecidos e células

-Northern Blotting
-RT-PCR (Reverse Trasncription Polymerase Chain Reaction)
-qRT-PCR (Real Time Quantitative Reverse Transcription Polymerase Chain Reaction)

Em seguida, dissertei sobre os métodos de análise global de RNAs em tecidos e células

-Microarranjos para análise de expressão gênica
-RNAseq em sequenciadores de próxima geração

#Em todos os pontos eu demonstrei conhecimento sobre as estratégias e as expressei na dimensão que uma prova deste tipo permite.

No tópico Identificação com a manutenção do contexto espacial na célula e tecido tratei dos seguintes métodos

-CISH - Chromogenic In situ Hybridization
-FISH - Fluorescent in situ hybridization
-RT-PCR in situ

#Detalhei sobre as sondas e métodos de obtenção dos resultados. Novamente, eu estou certo que aqui eu fui capaz de demonstrar conhecimento do tema e o expresse em linguagem acadêmica.

A dissertação foi fechada com o olhar para o futuro:

-Utilização de sondas para detectar RNA em células vivas
-RNAseq in situ (tema que aparece na literatura em 2013/2014)

Reitero que a estrutura e informações que apresentei demonstram sim, conhecimento do tema e sua manifestação, e que o fiz em linguagem acadêmica. A aderência ao tema pode ser facilmente verificada na literatura científica. A busca no banco de referências bibliográficas PUBMED utilizando os termos "RNA identification tissues cells" retorna 3927 referências. E, em sua grande maioria, pode-se identificar algum dos aspectos tratados em minha prova escrita. Abaixo uma amostra com três referências. Estes artigos estão em anexo.

Cell Tissue Res. 2008 Jun;332(3):381-91. doi: 10.1007/s00441-008-0606-8.
Sex-steroidal regulation of aromatase mRNA expression in adult male rat brain: a quantitative non-radioactive in situ hybridization study.
Zhao C1, Fujinaga R, Yanai A, Kokubu K, Takeshita Y, Watanabe Y, Shinoda K.

Hear Res. 2015 Jul;325:42-8. doi: 10.1016/j.heares.2015.03.008.
High quality RNA extraction of the mammalian cochlea for qRT-PCR and transcriptome analyses.
Vikhe Patil K1, Canlon B2, Cederroth CR3.

Nat Methods. 2008 Oct;5(10):877-9. doi: 10.1038/nmeth.1253.
Imaging individual mRNA molecules using multiple singly labeled probes.
Raj A1, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S.

O conhecimento demonstrado na dissertação em linguagem acadêmica reflete a experiência que eu tenho, orientando alunos em nível de Pós Graduação e em artigos publicados em revistas com revisão por pares e indexadas.

Orientações de Mestrado

Tainá Macherini Marques. Sequenciamento global de micrornas em soro de medula ossea ao diagnóstico e ao seguimento do tratamento de leucemia linfoide aguda. 2013. Dissertação (Mestrado em Ciências da Saúde) - Universidade de Brasília, Conselho Nacional de Desenvolvimento Científico e Tecnológico. **Orientador: Rinaldo Wellerson Pereira**. *Identificação de microRNAs por qRT-PCR

Getulio Pereira de Oliveira Junior. Análise de micrornas músculo específicos em frações plasmáticas antes e após corrida de meia maratona. 2012. Dissertação (Mestrado em Patologia Molecular) - Universidade de Brasília, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. **Orientador: Rinaldo Wellerson Pereira.** *Identificação de micrRNAs por qRT-PCR

Teses de Doutorado

Marcela Aparecida Chiabai. Genética e Genômica no estudo da resposta ao tratamento para Leucemia Linfoide Aguda. 2014. Tese (Doutorado em Ciências Genômicas e Biotecnologia) - Universidade Católica de Brasília, Conselho Nacional de Desenvolvimento Científico e Tecnológico. **Orientador: Rinaldo Wellerson Pereira.** *Neste trabalho de doutorado mRNAs foram identificados em células de pacientes com Leucemia Linfoide Aguda utilizando a metodologia de RNAseq.

Clarisa Pedrosa da Costa Gomes. Investigação de micrornas e mrnas na adapatação ao exercício aeróbio em ratos e humanos. 2013. Tese (Doutorado em Ciências Genômicas e Biotecnologia) - Universidade Católica de Brasília, Conselho Nacional de Desenvolvimento Científico e Tecnológico. **Orientador: Rinaldo Wellerson Pereira.** *microRNAs identificados por qRT-PCR; mRNA identificados por RNAseq e qRT-PCR

Artigos

CAMPOS, C. B. ; MARQUES, T. M. ; **Pereira, R.W**. ; SANDRIM, V. C. . Reduced circulating miR-196b levels is associated with preeclampsia. Pregnancy Hypertension: an international journal of women's cardiovascular health, v. 4, p. 11-13, 2014.

GOMES, C. P.C. ; OLIVEIRA JR, G. P. ; Madrid B ; ALMEIDA, JEESER ALVES ; Franco, O.L. ; **Pereira, Rinaldo W**. . Circulating miR-1, miR-133a, and miR-206 levels are increased after a half-marathon run. Biomarkers (London. Print), p. 1-5, 2014.

Artigos de Revisão

Gomes, Clarissa P. C. ; CHO, JI-HOON ; HOOD, LEROY ; FRANCO, OCTÁVIO L. ; **Pereira, Rinaldo W.** ; WANG, KAI . A Review of Computational Tools in microRNA Discovery. Frontiers in Genetics, v. 4, p. 1, 2013.

ETHERIDGE, ALTON ; Gomes, Clarissa P. C. ; **Pereira, Rinaldo W.** ; GALAS, DAVID ; WANG, KAI . The complexity, function and applications of RNA in circulation. Frontiers in Genetics, v. 4, p. 1, 2013.

ROCHA, L. A. ; Petriz, B. ; Borges, D.H. ; OLIVEIRA, Ricardo Jacó de ; ANDRADE, R. V. ; Domont, G ; **PEREIRA, R. W**. ; Franco, O.L. . High molecular mass proteomics analyses of left ventricle from rats subjected to differential swimming training. BMC Physiology (Online), v. 12, p. 11, 2012. *Neste trabalho nós utilizamos o qRT-PCR para identificar e quantificar a expressão do mRNA do gene da cadeia

pesada da alfa-miosina. O texto completo pode ser lido aqui: http://www.biomedcentral.com/1472-6793/12/11.

Uma experiência de 15 anos de trabalho com metodologias para identificação de RNA em células e tecidos e uma ampla revisão de literatura feita durante a preparação para este concurso público de provas e títulos sedimenta a certeza que a dissertação apresentada demonstra conhecimento e capacidade de expressá-lo em linguagem acadêmica.

Ao buscar pelas palavras mRNA e RNA no Curriculum Vitae (Plataforma Lattes) dos membros da banca examinadora (Prof. Luiz Augusto Batista Brito; Profa. Irmtraut Araci Hoffmann Pfrimer; Prof. Anilton Cesar Vasconcelos; Prof. Antonio Carlos Alessi e Prof. Sony Dimas Bicudo), somente o CV do Prof. Anilton Cesar Vasconcelos apresenta o termo mRNA. Este termo aparece em um projeto que propõe a identificação do mRNA do gene caspase em baço e timo de ratos utilizando a técnica de RT-PCR. Esta pouca familiaridade da banca com o tema, como demonstrado pela busca na Plataforma Lattes, a despeito de sua qualificação em suas áreas de atuação, pode ter acarretado a construção de uma chave de resposta com itens pouco abrangentes para a diversidade e novidade do tema. E, desta forma, ter redundado em minha eliminação.

Durante a sessão pública de divulgação de resultado, eu não fui informado sobre minha nota e tampouco sobre a chave de resposta utilizada para correção de minha prova. A não publicidade da chave de resposta compromete sobremaneira a manifestação do contraditório e é incompatível com Princípio da Legalidade, norteador da atividade administrativa.

Diante do exposto, requer-se:

1. O encaminhamento deste recurso para a Comissão previamente constituída, com pelo menos três membros, previamente indicados pelo Conselho Diretor da Unidade para que conheça e decida sobre o presente recurso no prazo regimental, previsto no parágrafo único do artigo 16 da Resolução Conjunta - Consuni/CEPEC n 02/2013.

2. Que a Banca Examinadora dê publicidade à “chave de resposta” exigida ao ponto sorteado pelo candidato RINALDO WELLERSON PEREIRA, mesmo que na Resolução Conjunta - Consuni/CEPEC n 02/2013, bem como nas Normas Complementares ao Edital 73/2014, não vinculem expressamente a resposta da prova escrita a um padrão de “chave de resposta”;

3. Que a Banca Examinadora, em sessão pública, informe ao candidato RINALDO WELLERSON PEREIRA sua nota na prova escrita;

4. Que seja dada publicidade ao espelho de resposta da Prova Escrita do Candidato pela página SISCONCURSOs da UFG;

5. Seja dado provimento ao presente recurso determinando que a prova escrita do candidato RINALDO WELLERSON PEREIRA seja avaliada conforme estabelecido no artigo 20 da Resolução Conjunta - Consuni/CEPEC n 02/2013, bem como nas Normas Complementares ao Edital 73/2014;

6. A reintegração do candidato RINALDO WELLERSON PEREIRA ao concurso público para as etapas de Prova Oral na forma de Conferência Pública e para a Defesa do Memorial.

7. Que seja dada publicidade a este recurso

Nestes termos,
Pede deferimento

Goiânia-GO, 27 de Maio de 2015.

Rinaldo Wellerson Pereira

UNIVERSIDADE FEDERAL DE GOIÁS
ESCOLA DE VETERINÁRIA E ZOOTECNIA
(Cx. Postal 131 - Campus II, CEP: 74001-970 Tele/Fax: (062) 3521-1566 / 1565).

RESULTADO DA PROVA ESCRITA

Concurso público de provas e títulos para Professor Efetivo Titular-Livre da carreira do Magistério Superior da Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, regime de trabalho: 40h/DE, na área de Biologia Celular e Molecular aplicadas à Ciência Animal, conforme o que consta no processo nº. 23070.022340/201477, Edital nº. 73/2014 e na Resolução Conjunta CONSUNI/CEPEC Nº. 02/2013.

ÁREA: Biologia Celular e Molecular aplicadas à Ciência Animal
UNIDADE ACADÊMICA: Escola de Veterinária e Zootecnia

Conforme item III, alínea "a" das normas complementares do edital 73/2014, publicado no D.O.U. em 21/11/2014, *a prova escrita tem o* ***caráter eliminatório***.

CANDIDATO	RESULTADO
Cesario Bianchi Filho	Não Compareceu
Rinaldo Wellerson Pereira	Eliminado

OBS: Resultado divulgado em sessão pública, realizada as15h05min, do dia 26 de maio do ano dois mil e quinze na Escola de Veterinária e Zootecnia, de acordo com Art. 15º da Resolução Conjunta CONSUNI/CEPEC Nº 02/2013.

Diretoria da Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, 16h20min do dia 26 de maio do ano dois mil e quinze.

Prof. Dr. Marcos Barcellos Café
DIRETOR DA ESCOLA DE VETERINÁRIA E ZOOTECNIA DA UFG

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Research paper

High quality RNA extraction of the mammalian cochlea for qRT-PCR and transcriptome analyses

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ABSTRACT

Molecular investigations of the hearing organ, the cochlea, have been hampered due to the difficulty of isolating pure RNA and in quantities sufficient enough for quantitative real-time RT-PCR or microarray analysis. The complex architecture of the cochlea, the presence of liquids, bone and cartilage tissue, are a major hurdle in obtaining contamination-free RNA to a level that does not affect downstream applications. Here, we present a protocol to extract RNA from the mouse cochlea, with yields and quality suitable for real-time RT-PCR or Affymetrix labeling. In contrast to current methods, such as TRIZOL or column-based extraction, this protocol combines the two and, within 4 h, yields a 2 μg of total RNA from a single pair of adult mouse cochleae. This protocol allows the isolation of RNA molecules from the mammalian cochlea providing access to whole-transcript expression analyses.

1. Introduction

Genome-wide expression profiling has brought considerable knowledge on biological processes. However, in tissues with bone such as the cochlea, limited amount of cellular mass, cellular heterogeneity (comprised of blood vessels, epithelial cells, mesenchymal cells, neurons, support cells and sensory hair cells), matrix compartments, have hampered the use of such technologies. Hence, progress in understanding transcriptional networks underlying adult auditory functions has been slow.

Several commercial kits (Invitrogen TRIZOL, Qiagen RNeasy Micro Kit) have been used for the extraction of ribonucleic acids from the mammalian cochlea (reviewed by Hertzano and Elkon, 2012). Nearly a fourth of all transcriptome studies of the inner ear were performed on adult cochleae using TRIZOL (D'Souza et al., 2008; Gong and et al., 2006; Gu et al., 2006; Shah et al., 2009; Someya et al., 2007) or Qiagen kits (Chen and Corey, 2002; Hildebrand and et al., 2005; Huang and et al., 2011; Im et al., 2007; Jabba and et al., 2006; Liu et al., 2004; Morris and et al., 2005; Yoshimura and et al., 2014; Son and et al., 2012; Smeti et al., 2012). Albeit efficient when studying the neonatal cochlea, these methods have limitations when applied to the adult cochlea due to limited recovery of RNA, and high yields of contaminants that preempt the downstream use of genome-wide transcriptome approaches or real-time RT-PCR.

Nucleic acid purity is often evaluated by ultraviolet absorbance. Absorbance at 260 nm is used to assess the amount of nucleic acid in the sample, whereas the amount of protein and organic contaminants is evaluated at 280 and 230 nm respectively. Using *A*260/*A*230 and *A*260/*A*280 ratios, one is able to estimate the purity of the RNA extraction. Typical requirements for *A*260/*A*280 are 1.8–2.2, while *A*260/*A*230 should be >2. Bioanalyzer chips from Agilent technologies offer a miniaturized version of agarose gels, in which the intensity of the major ribosomal RNA (rRNA) bands can be quantified. A ratio of 2:1 for 28S/18S rRNA is representative of good-quality RNA. As RNA degrades, peak heights of 28S and 18S rRNA decrease, while smaller or degraded peaks become apparent. The Bioanalyzer also provides a value of RNA integrity (RIN) from 0 to 10, which takes into account the whole electrophoretic trace of the sample, with 10 being the maximum RNA integrity.

Here, we show that adult cochleae extracted with Qiagen RNeasy Micro kit show high level of organic contaminants at 230 nm in 3 out of 8 extractions, providing an average ratio of A_{260}/A_{230} nm below zero as measured with a Nanodrop ND-1000 spectrophotometer (Fig. 2). With the multiple successful RNA extractions (>200) we have performed in the past with this kit on multiple types of tissues (e.g. liver, adipose tissue, muscle, testis and brain) (Cederroth and et al., 2007a; Cederroth and et al., 2008;

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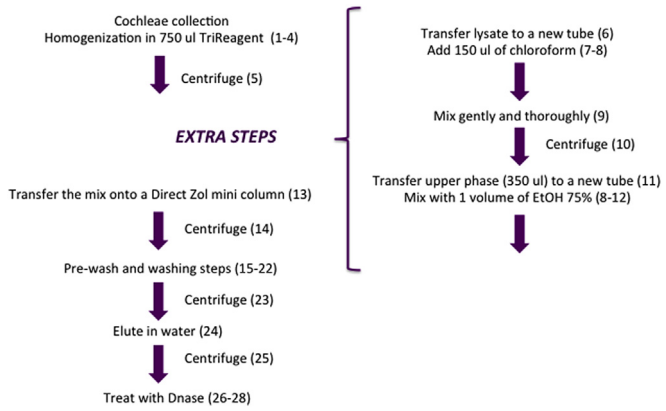


Fig. 1. Flow diagram to summarize the major steps in cochlear RNA extraction using the CRC protocol.

Cederroth and et al., 2007b; Cederroth and et al., 2010; Nef and et al., 2005; Pitetti and et al., 2009; Grad and et al., 2010), we could hardly explain such observations. Potentially, residual bone debris collected even after centrifugation, could affect membrane efficacy. However, we never found such low values when using Zymoresearch's Direct-Zol RNA mini prep kit on more than a hundred cochlear samples (Meltser and et al., 2014). Analysis through Bioanalyzer chips (Agilent technologies) indicated low RIN of an average of 3.93 ± 0.32. These low RIN values were independent of the 230 contaminants. Qiagen extractions of the cochlea also yielded low values of 28S/18S (0.53 ± 0.03) indicating mRNA degradation has occurred (Fig. 3 and Table 1). In comparison, control Qiagen extractions of the liver show much greater quality (Table 1), and confirmed proper handling. It is known that guanidinium thiocyanate-phenol-chloroform (Trizol) extraction alone yields high levels of phenol contaminants (measured at 230 nm) that are incompatible with hybridizations. The recent development of ZymoResearch kits, such as Direct-Zol RNA mini prep, partly circumvents this issue by having all ethanol washes performed on column immediately after the lysis with Trizol. Surprisingly, as we tested different Trizol providers, only the TRI-Reagent® provided by Zymoresearch was efficient on their columns unlike TrizolRegeant (Invitrogen) or Trizol LS (Invitrogen) with which no bands could be observed on the Bioanalyzer chip in spite of similar nucleotide output in terms of quantity (data not shown). Hence, for sake of clarity, we only provide values using the Trizol (TRI-Reagent®) originating from ZymoResearch kit. Although organic contaminants were less abundant (230 nm) and RNA degradation less important (28S/18S) than with Qiagen RNeasy Micro kit (Figs. 2, 3), A_{260}/A_{280} ratios were not as good and the RIN was equally low (Table 1). We hypothesized that still some residual bone or matrix component from the cochlea, in spite of our careful pipetting, could affect both commercial columns' binding affinity to RNA.

The protocol described here (CRC protocol) combines the advantage of both Trizol and column purification by using the Qiagen RNeasy Micro kit or the Direct-Zol RNA mini prep (ZymoResearch) kit, but adding an extra step with chloroform phase separation, which aqueous phase is precipitated with one volume of ethanol 70% prior being deposited on the column, and further processed according to the manufacturer's protocol. This step ensured that the RNA from the aqueous phase was free of protein and organic contaminants when purified on column. Because DNA contamination causes false–positive reactions in amplification processes such as RT-PCR, we performed DNase treatment. However, in order to allow comparisons between all tested conditions, we performed the treatment with DNaseI (Invitrogen) after eluting the RNA with the same volume of Tris–EDTA buffer (30 μl), rather than on column as suggested by the manufacturers. With this protocol, we found that TriRegeant extraction followed by chloroform separation and Direct-Zol RNA mini prep column purification achieved the best RNA quality (Table 1).

1.1. The CRC protocol

The CRC protocol (Fig. 1) allows the recovery of about 2 ug of RNA from a single pair of adult cochleae, suitable for real-time RT-PCR analyses, NanoString nCounter or GeneChip technologies. The CRC protocol provides high yield of RNA from a single pair of cochleae, free of DNA, and limited contamination. The CRC protocol is designed to process 4 pairs of cochleae at a time and provide high yield and high quality RNA.

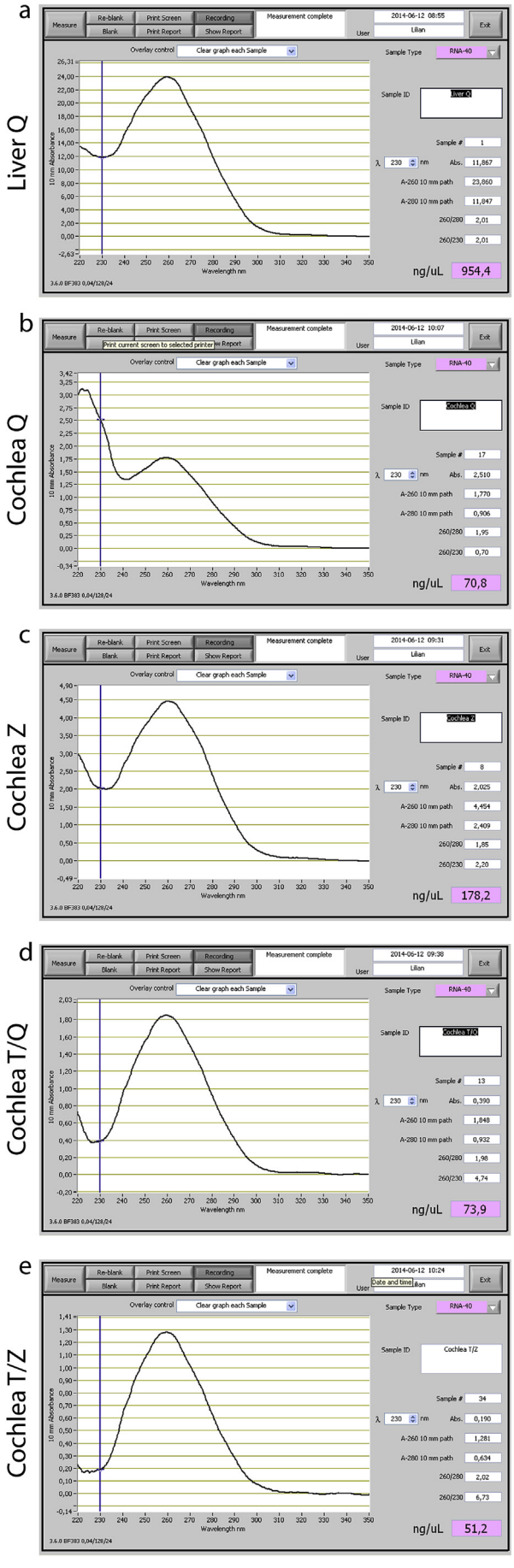
When compared to Qiagen RNeasy Micro kit or Direct-Zol RNA mini prep kits, the RNA purified with the CRC protocol consistently yields purer RNA, typically with A_{260}/A_{280} ratios of 1.96–2.06 optical densities. Absorbance ratios of 260/230 > 2 are required for *in vitro* transcription in Affymetrix experiments and labeling reactions. This was achieved using the CRC protocol, whereas with column based procedures such as Qiagen RNeasy Micro kit an average ratio of ~5 was obtained (Fig. 2).

The integrity and the size of distribution of purified RNA were verified on Bioanalyzer (Agilent) microfluidic electrophoresis chips. The Direct-Zol RNA mini prep kit was more efficient than the Qiagen RNeasy Micro kit in providing contaminant-free RNA but when applying the CRC protocol to Direct-Zol RNA mini prep columns, we achieved greater 28S/18S ratios (1.41 ± 0.13) and higher RIN (6.86 ± 0.11). In comparison, RNA extracts free of impurities, as achieved when extracting RNA from cultured cell lines, reach a maximum RIN of 10. Table 1 summarizes the A_{260}/A_{280} and A_{260}/A_{230} ratios obtained with a Nanodrop ND-1000 spectrophotometer (shown in Fig. 2), and the RIN and the 28S/18S ratios obtained with a Bioanalyzer chip (Fig. 3). Without further purification, the CRC protocol in combination with Direct-Zol RNA mini prep columns is directly applicable for use in real-time RT-PCR (Fig. 4), Nanostring nCounter (Fig. 5) or Affymetrix (Fig. 6) probe labeling reactions. The suitability of this protocol for library constructions or rapid amplification of cDNA ends, has not been tested. Even though silica columns provide clear cytoplasmic RNA, we observe it has excluded smaller molecular weight RNA species, such as miRNA and other small RNA species. Specific kits for small RNA isolation might be required.

Overall, the purified cochlear RNA is suitable for downstream applications including northern blot analysis, hybridization procedures (NanoString nCounter and Affymetrix), reverse transcription and quantitative real-time RT-PCR without further processing. To date, our lab successfully isolated total RNA from the mouse cochlea using the CRC protocol coupled to Direct-Zol RNA mini prep purification for real-time RT-PCR on more than 100 cochlear pairs, for NanoString nCounter hybridizations and Affymetrix GeneChip hybridizations on more than 24 cochlear pairs (Cederroth, unpublished results).

2. Reagents

- TRI-Reagent® (ZymoResearch, cat. no. R2050-1-50) **Caution:** TRI-Reagent® is toxic and hazardous if it is ingested, inhaled or if it comes into contact with the skin or eyes.



- Chloroform (Sigma, C2432) **Caution:** Chloroform is hazardous if it is ingested, inhaled or if it comes into contact with the skin or eyes.
- Ethanol **Caution:** Both the liquid and the vapor forms are flammable. This reagent causes severe irritation of the eyes and moderate irritation of the skin.
- Liquid Nitrogen **Caution:** Contact with liquid or vapors can cause tissue damage through frostbite. The release of nitrogen gas causes oxygen concentrations to drop below 19.5%, and thus can cause suffocation.

  Wear safety goggles, gloves and a lab coat when handling these reagents and work in a fume hood.
- DNaseI (Invitrogen, 18068-015)

3. Equipment

- Magna Lyser (Roche)
- Screw cap tubes (StarLab, E1420-2641)
- 5/32″ Chrome steel balls from bearings (Aisi 52100)
- Safe seal 2 ml microtubes (Sarstedt, 72.695.500)
- 1.5 ml microtubes (Sarstedt, 72.690.001)
- Magnet (Promega 2)
- Direct-Zol RNA mini prep kit (ZymoResearch, R2052)
- Cold centrifuge (Eppendorf, 5424 R)
- Room temperature centrifuge (Eppendorf, 5415 D)
- Thermomixer (Eppendorf)
- Vortex (Scientific industries)
- RNase-free microcentrifuge tubes (Sigma–Aldrich, cat. no. Z717533)
- Bioanalyzer (Agilent, Santa Clara, CA, Model 2100). Alternatively, agarose gel electrophoresis system can be used instead of Bioanalyzer microfluidics-based platform
- Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

4. Reagent setup

4.1. Tris–EDTA (TE) buffer

For more accurate evaluation with the Nanodrop spectrophotometer, TE buffer is recommended. Add 1 ml of 1 M Tris–HCl (pH 8.0) and 0.2 ml of 1 M EDTA (pH 8.0) to 98.8 ml of dH2O. Filter and autoclave the solution. The solution can be stored in a tightly sealed glass bottle at room temperature up to a year.

5. Procedure

- **1. Tissue collection and homogenization** (**Timing: 1 h**). Transfer the frozen tissue from a −80 °C freezer to liquid nitrogen to prevent thawing prior to the next step. **Critical:** Collection of the tissue is very important for the quality of the RNA isolation. Tissues have to be dissected in cold buffer, or RNAlater in order to diminish RNA degradation. RNAlater was not used in these experiments, but its inclusion in the protocol could further increase RNA quality. Tissues should be frozen immediately in liquid nitrogen after collection and should be kept at −80 °C. Tissues should not be thawed prior to the addition of homogenization buffer.

Fig. 2. Spectrophotometry. Liver RNA extracted with Qiagen RNeasy Micro kit (a), adult cochlear RNA isolated with Qiagen RNeasy Micro kit (b), or Direct-Zol RNA mini prep (c), RNA from adult cochleae lysed with TRI-Reagent®, followed by chloroform separation and by purification on Qiagen RNeasy Micro columns (d), or Direct-Zol RNA mini columns (e). Note the high level of impurities at 230 nm absorbance after extraction with the Qiagen RNeasy Micro kit (b).

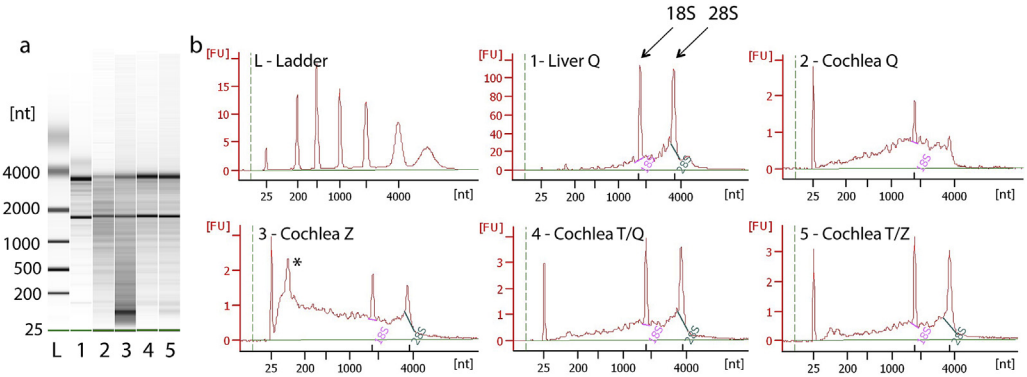


Fig. 3. The quality of the RNA isolated using various methods measured with an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip. (a) The first lane contains the RNA ladder (L), sample lane (1) contains Liver RNA extracted with Qiagen RNeasy Micro kit, sample lanes 2–5 contain adult cochlear RNA isolated with (2) Qiagen RNeasy Micro kit, (3) Direct-Zol RNA mini prep, (4) TRI-Reagent® lysis with chloroform separation followed by purification on Qiagen RNeasy Micro columns, (5) TRI-Reagent® lysis with chloroform separation followed by purification on Direct-Zol RNA mini columns. (b) Electropherogram of the samples helps determining the intensity of each band on the gel, mainly the 28S and 18S ribosomal bands. Note the presence of small molecular weight RNA (asterisk) in sample (3), which is absent in all other samples. [nt] = nucleotide size; [FU] = fluorescence unit.

2. Place one chrome steel ball per secured cap tube and add 750 μl of Trizol for a pair of cochleae (more cochleae can be added if needed, up to a dozen).
3. Add the frozen cochleae, and immediately homogenize with the MagnaLyser at 5000 rpm for 10 s.
4. Vortex 5 s and remove beads from the tube with a magnet.
5. Centrifuge the sample at 12,000*g* for 5 min at 4 °C to remove bone debris.
6. Transfer the lysate to a safe seal 2 ml microtube.
   Troubleshooting – **Pause point:** If you cannot immediately proceed with the RNA extraction protocol, homogenized tissue can be safely stored in TRI-Reagent® at −80 °C for 3 months.
7. Let the lysate rest for 5 min at room temperature.

- **8. Trizol-Chloroform aqueous phase separation** (**Timing: 45 min).** Add 150 μl of chloroform for every 750 μl of Trizol and shake vigorously for 15 s.
  Caution: Chloroform- and phenol-containing steps should be handled in the fume hood wearing protective clothing.

9. Let the sample mixture rest for 5 min at room temperature.
10. Centrifuge the sample mixture at 12,000*g* for 15 min at 4 °C.
11. Transfer 350 μl of the aqueous phase to a clean RNase-free microcentrifuge tube.

- **12. Column purification (Timing: 30 min).** Add 350 μl of ethanol 70% to the aqueous phase and pipet gently up and down 2–3 times.

13. Transfer 700 μl of the sample mixture to a minicolumn placed in a 2 ml collection tube from the Direct-Zol RNA mini prep kit.
14. Centrifuge for 30 s at 12,000*g* at RT and discard the flow-through.
15. Add 400 μl of Pre-wash buffer (ZymoResearch) to the column.
16. Centrifuge for 30 s at 12,000*g* at RT and discard the flow-through.
17. Add 400 μl of Pre-wash buffer (ZymoResearch) to the column.
18. Centrifuge for 30 s at 12,000*g* at RT and discard the flow-through.
19. Transfer the column to a new collection tube and add 700 μl Wash buffer (ZymoResearch).
22. Centrifuge for 2 min at 12,000*g* at RT and discard the flow-through.
23. Place the column in a new collection tube, open the lid of the columns, and centrifuge for 5 min to ensure no ethanol carry over.
24. Place the column in a clean 1.5 ml RNase-free micro-centrifuge tube, add 30 μl of RNase free water or TE. **Critical:** TE provides a more accurate evaluation of RNA abundance due to more optimal pH, however it is not recommended when performing downstream measures such as qRT-PCR or chip arrays.
25. Centrifuge for 1 min at 12,000*g* at RT.
    Pause point: Store the RNA at −80 °C. RNA is stable for 1 year.

- **26. DNase treatment** (**Timing: 30 min).** Add 1.5 μl of 10X buffer and 1 μl of DNaseI (Invitrogen) per sample tube and incubate 15 min at RT

27. Add 1 μl of EDTA and incubate 10 min at 65 °C.
28. Place on ice or store at −80 °C.
29. Measure RNA concentration (NanoDrop)
    Troubleshooting – **Pause point:** DNase treatment can be performed during the column purification in order to ensure minimal EDTA contamination and potential interference with downstream applications. Here, because the DNase treatment (buffers and DNases) from Zymoresearch and

Table 1
Adult cochlea RNA quality measurements. Mean ± SEM of 4–8 samples. (1) Liver Q is liver RNA extracted with Qiagen RNeasy Micro kit, (2) Cochlea Q is adult cochlear RNA isolated with Qiagen RNeasy Micro kit, (3) Cochlea Z is adult cochleae RNA extracted with Direct-Zol RNA mini prep, (4) Cochlea T/Q and (5) T/Z are RNA from adult cochleae lysed with TRI-Reagent®, separated with chloroform and followed by purification on Qiagen RNeasy Micro columns or Direct-Zol RNA mini columns, respectively.

	(1) Liver Q	(2) Cochlea Q	(3) Cochlea Z	(4) Cochlea T/Q	(5) Cochlea T/Z
A260/280	2.05 ± 0.00	2.05 ± 0.01	1.91 ± 0.00	1.96 ± 0.02	2.03 ± 0.01
A260/230	1.94 ± 0.13	−5.12 ± 3.41	2.74 ± 0.23	2.62 ± 1.03	8.03 ± 2.00
RIN	8.30 ± 0.15	3.93 ± 0.32	4.72 ± 0.58	7.00 ± 0.18	6.86 ± 0.11
28S/18S	1.35 ± 0.03	0.53 ± 0.03	1.24 ± 0.12	1.12 ± 0.08	1.41 ± 0.13

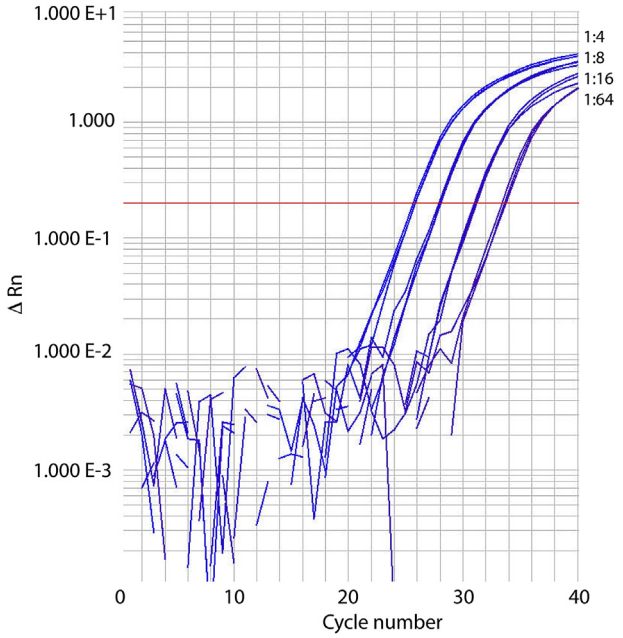


Fig. 4. Amplification plot showing the expression level of *eeat1* gene (*Glast*) measured by Sybergreen qPCR. The cDNA was prepared by using total RNA isolated from cochleae of adult CBA mice with the procedure described in this paper. Primers were designed using Primer 3 software. The reactions were set up with serial dilutions of the cDNA, (d1) 1:4, (d2) 1:8, (d3) 1:16 and (d4) 1:64. Note the uniform amplification of the 1:64 triplicates at 34 cycles.

Qiagen are different (the one from Zymoresearch decreases the total RNA yield), we performed the above DNase treatment after the elution step in order to allow comparisons of column efficacy without the bias generated by this step. We recommend DNase treatment on column when using column kits. This also avoids prolonged exposure of RNA to room temperature.

- **30. Measuring RNA concentration. (Timing: 10 min**). Thaw on ice and determine the RNA concentration spectrophotometrically by measuring the absorbance at 230, 260 and 280 nm. The purity of the RNA can be estimated by calculating A_{260}/A_{280} ratios. Pure RNA has an A_{260}/A_{280} ratio between 1.8 and 2.2. A_{260}/A_{230} ratios help determining whether organic impurities are present and should be > 2. **Critical step** To measure RNA concentration with a Nanodrop spectrophotometer, the samples must be diluted to 2–3000 ng μl^{-1}. Here, you may use 1.5–2 μl of the cochlear RNA sample directly for spectrophotometric measurements with the ND-1000 for best results (Fig. 2). **Troubleshooting** (Table 2).
- **31. Checking RNA quality by Bioanalyzer microfluidic electrophoresis chips. (Timing**: **30 min**). For total RNA, RNA 6000 Nano chip can be used. Prepare the Bioanalyzer microfluidic electrophoresis chips according to the manufacturer's protocol (http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90034_KitRNA6000Nano_ebook.pdf). After determining the RNA concentration spectrophotometrically, use the remaining RNA to verify its quality with an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip.

  Load 1 μl of the sample onto the gel, the RNA concentration should be between 25 and 500 ng μl^{-1} Fig. 3 shows the Bioanalyzer microfluidic electrophoresis chip and individual graphic presentations of RNA isolated with various RNA isolation protocols.

6. Timing

Steps 1–8: Tissue collection and homogenization, 1 h for 4 pairs of cochleae.
Steps 8–11: Trizol-Chloroform aqueous phase separation, 45 min.
Steps 12–25: Column purification, 30 min.
Steps 26–29: DNase treatment, 30 min.
Steps 30: Measuring RNA concentration, 1 h for 4 pairs of cochleae.
Steps 31: Checking RNA quality by Bioanlyzer microfluidic electrophoresis chip, 30 min.

7. Anticipated results

The CRC protocol presented here yields high-purity, DNA-phenol- and organic-free RNA from the adult cochlea (Table 1). Comparison with other standard methods shows that RNA is of greater quality and free of contaminants (Figs. 2, 3). Data from the Bioanalyzer indicates that samples processed with the CRC protocol are devoided of small molecular weight RNA (Fig. 3). Nonetheless, protein and phenol contamination is low, RNA integrity is greater

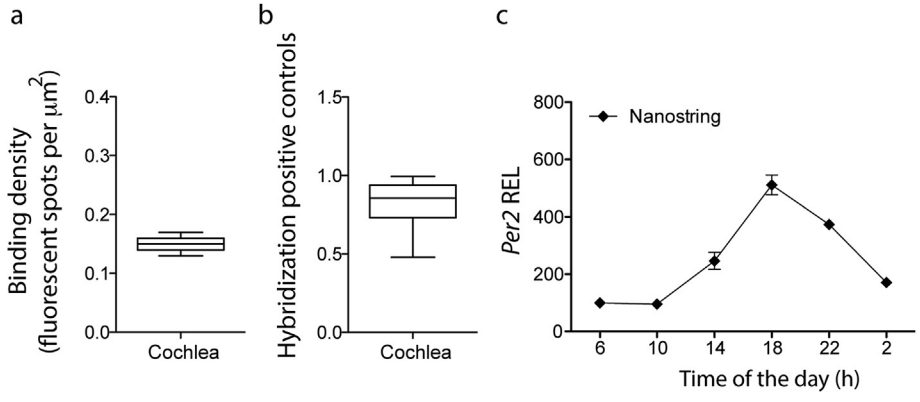


Fig. 5. NanoString nCounter hybridization quality control. 100 ng of cochlear RNA isolated with the CRC protocol coupled to Direct-Zol RNA mini prep columns purification was hybridized on NanoString nCounter chips. (a) Quality of hybridization binding is shown and should be below the value 1. (b) Positive controls values provide an insight on the homogeneity of sample quality and purity, which should not be below the value 0.3. (c) *Per2* gene expression at different time points throughout the day performed with Nanostring nCounter (black diamonds). REL = relative expression level, N = 4 pairs of cochleae per time point. Note the low variability of the biological replicates.

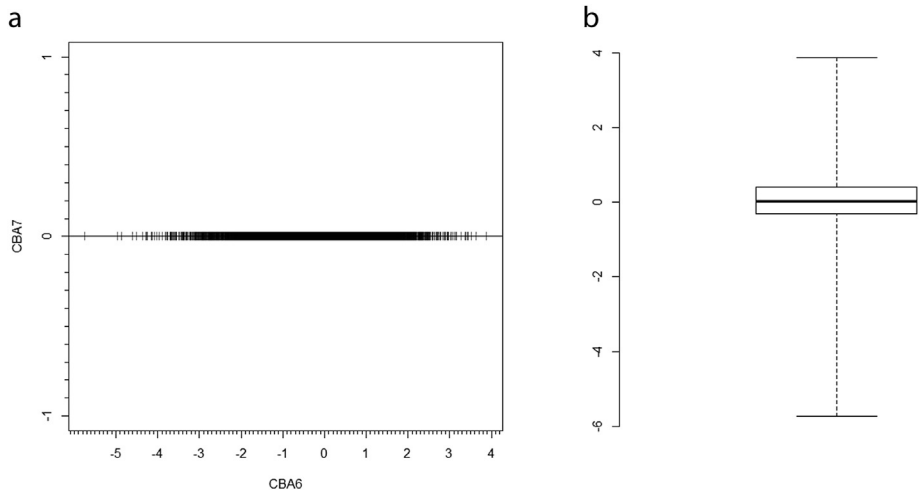


Fig. 6. Results of Affymetrix hybridization statistics. The RNA isolated with the CRC protocol followed by Direct-Zol RNA mini prep columns purification was used directly for probe labeling and hybridization on two different Mouse Gene 2.0 ST Arrays. The results of the hybridizations were compared to one another to evaluate whether differences could be observed RNA impurities. The results from the one of the chips were used as a baseline in the analysis. (a) Scatter plot analysis of hybridizations and (b) ratio of the signal distribution of the two chips.

Table 2
Troubleshooting table.

Step	Problem	Possible reason	Solution
1–3	RNA degradation	Cochleas are kept at RT during their dissection.	Immediately isolate the inner ear and dissect the cochlea in cold PBS 1X or RNAlater.
		Too much delay between tissue isolation and RNA extraction.	Immediately add the lysis buffer to the isolated cochlea and pulverize with the Magna Lyser.
		Potential RNase contamination in glass, plasticware or solutions.	Be sure that all plasticware free of RNase. Never use glassware.
		Temperature for tissue storage before homogenization is not low enough.	If cochlear lysis cannot be performed immediately, freeze the tissue in liquid nitrogen and store at −80 °C.
26	RNA degradation	DNase treatment after the elution exposes RNA to room temperature for too long.	Perform DNase treatment on column as recommended by the manufacturer.
1–28	RNA degradation	Intervention of multiple staff causing variations in handling and precautions.	Ensure optimal training to maintain homogeneous skills among laboratory staff.

than with other tested kits (Fig. 3) and the ratio of 26s to 18s indicates less RNA degradation (Fig. 3). The protocol is performed in 4 h and the total RNA isolated can be used for Real-Time PCR (Fig. 4), NanoString nCounter (Fig. 5) or Affymetrix Gene Chip analyses (Fig. 6). The quantitative real-time PCR from the *eeat1* gene shows efficient amplification, even at low dilutions (Fig. 4). NanoString nCounter analyses of cochleas sampled at different time points throughout the day shows low variability among samples with robust circadian expression profiles (Fig. 5) similar to those published using qRT-PCR (Meltser and et al., 2014). Affymetrix analyses show very high quality and binding efficacy (Fig. 6). Generally, with one pair of cochlea, 2 μg of total RNA can be isolated.

Author contribution and competing financial interest

B.C and C.R.C. conceived the project and supervised the project. K.V.P. and C.R.C. performed experiments and analyzed data. C.R.C. wrote the paper. The authors declare they have no competing financial interests.

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References

Cederroth, C.R., et al., 2007. Estrogen receptor alpha is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism. Endocrinology 148, 5507–5519.
Cederroth, C.R., et al., 2007. A phytoestrogen-rich diet increases energy expenditure and decreases adiposity in mice. Environ. Health Perspect. 115, 1467–1473.
Cederroth, C.R., et al., 2008. Dietary phytoestrogens activate AMP-activated protein kinase with improvement in lipid and glucose metabolism. Diabetes 57, 1176–1185.
Cederroth, C.R., et al., 2010. Potential detrimental effects of a phytoestrogen-rich diet on male fertility in mice. Mol. Cell. Endocrinol. 321, 152–160.

Chen, Z.Y., Corey, D.P., 2002. An inner ear gene expression database. J. Assoc. Res. Otolaryngol. 3, 140–148.
D'Souza, M., Zhu, X., Frisina, R.D., 2008. Novel approach to select genes from RMA normalized microarray data using functional hearing tests in aging mice. J. Neurosci. Methods 171, 279–287.
Gong, T.W., et al., 2006. Age-related changes in cochlear gene expression in normal and shaker 2 mice. J. Assoc. Res. Otolaryngol. 7, 317–328.
Grad, I., et al., 2010. The molecular chaperone Hsp90alpha is required for meiotic progression of spermatocytes beyond pachytene in the mouse. PLoS One 5, e15770.
Gu, F.M., Han, H.L., Zhang, L.S., 2006. Effects of vasopressin on gene expression in rat inner ear. Hear. Res. 222, 70–78.
Hertzano, R., Elkon, R., 2012. High throughput gene expression analysis of the inner ear. Hear. Res. 288, 77–88.
Hildebrand, M.S., et al., 2005. Expression of the carrier protein apolipoprotein D in the mouse inner ear. Hear. Res. 200, 102–114.
Huang, M., et al., 2011. Overlapping and distinct pRb pathways in the mammalian auditory and vestibular organs. Cell Cycle 10, 337–351.
Im, G.J., Jung, H.H., Chae, S.W., Cho, W.S., Kim, S.J., 2007. Differential gene expression profiles in salicylate ototoxicity of the mouse. Acta Otolaryngol. 127, 459–469.
Jabba, S.V., et al., 2006. Macrophage invasion contributes to degeneration of stria vascularis in Pendred syndrome mouse model. BMC Med. 4, 37.
Liu, X., Mohamed, J.A., Ruan, R., 2004. Analysis of differential gene expression in the cochlea and kidney of mouse by cDNA microarrays. Hear. Res. 197, 35–43.
Meltser, I., et al., 2014. TrkB-mediated protection against circadian sensitivity to noise trauma in the murine cochlea. Curr. Biol. 24, 658–663.
Morris, K.A., et al., 2005. Differential expression of genes within the cochlea as defined by a custom mouse inner ear microarray. J. Assoc. Res. Otolaryngol. 6, 75–89.
Nef, S., et al., 2005. Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development. Dev. Biol. 287, 361–377.
Pitetti, J.L., et al., 2009. Insulin receptor and IGF1R are not required for oocyte growth, differentiation, and maturation in mice. Sex. Dev. 3 (5), 264–272.
Shah, S.M., Kang, Y.J., Christensen, B.L., Feng, A.S., Kollmar, R., 2009. Expression of Wnt receptors in adult spiral ganglion neurons: frizzled 9 localization at growth cones of regenerating neurites. Neuroscience 164, 478–487.
Smeti, I., Assou, S., Savary, E., Masmoudi, S., Zine, A., 2012. Transcriptomic analysis of the developing and adult mouse cochlear sensory epithelia. PLoS One 7, e42987.
Someya, S., Yamasoba, T., Prolla, T.A., Tanokura, M., 2007. Genes encoding mitochondrial respiratory chain components are profoundly down-regulated with aging in the cochlea of DBA/2J mice. Brain Res. 1182, 26–33.
Son, E.J., et al., 2012. Developmental gene expression profiling along the tonotopic axis of the mouse cochlea. PLoS One 7, e40735.
Yoshimura, H., et al., 2014. Deafness gene expression patterns in the mouse cochlea found by microarray analysis. PLoS One 9, e92547.

Imaging individual mRNA molecules using multiple singly labeled probes

Arjun Raj[1], Patrick van den Bogaard[2], Scott A Rifkin[1], Alexander van Oudenaarden[1] & Sanjay Tyagi[2]

We describe a method for imaging individual mRNA molecules in fixed cells by probing each mRNA species with 48 or more short, singly labeled oligonucleotide probes. This makes each mRNA molecule visible as a computationally identifiable fluorescent spot by fluorescence microscopy. We demonstrate simultaneous detection of three mRNA species in single cells and mRNA detection in yeast, nematodes, fruit fly wing discs, and mammalian cell lines and neurons.

As it is becoming increasingly apparent that gene expression in individual cells deviates substantially from the average behavior of cell populations[1], new methods that provide accurate integer counts of mRNA copy numbers in individual cells are needed. Ideally, such methods should also reveal the intracellular locations of the mRNAs, as mRNA localization is often used by cells to spatially restrict the activity of proteins[2]. One candidate for such a method is *in situ* hybridization followed by microscopic analysis[3,4]. A conventional practice is to link probes to enzymes that catalyze chromogenic or fluorogenic reactions[5]. However, because the products of these reactions are small molecules or precipitates that diffuse away from the probe, the location of the target molecule is not precisely determined. Conversely, probes labeled directly with a few fluorophores maintain spatial resolution, but the sensitivity that can be achieved is relatively poor.

To circumvent these problems, a variant of fluorescence *in situ* hybridization (FISH) procedure has been developed that is sensitive enough to detect single mRNA molecules[6]. In this procedure, 5 oligonucleotide probes, each about 50 nucleotides long and labeled with 5 fluorophore moieties, are hybridized to each mRNA target, which then becomes visible as a diffraction-limited fluorescent spot. Although these probes have been used successfully[7], the system has not been widely adopted. One reason for this is difficulty in synthesizing and purifying heavily labeled oligonucleotides: the amine groups used for coupling fluorophores to the probe are prone to loss, and it is hard to purify fully coupled probes from partially coupled ones[8]. Also, when some fluorophores are present in multiple copies on the same oligonucleotide, they interact with each other, altering the hybridization characteristics of the oligonucleotides and resulting in severe self-quenching[9].

Another issue with the use of small numbers of heavily labeled probes is that the signals are more prone to variability. For instance, when using 5 fluorescent probes targeted to a single mRNA, the researchers had estimated that the majority of the fluorescent spots observed have intensities corresponding to the presence of only 1 or 2 probes[6]. This makes it difficult to unambiguously identify all the fluorescent spots as mRNA molecules as it is impossible to determine whether the detection of an individual probe arises from legitimate binding to the target mRNA or from nonspecific binding.

To address these issues, we reasoned that by taking advantage of the high throughput of 96-position DNA synthesizers, one could synthesize a large number of probes and reliably label them with a single fluorophore moiety at their 3′ termini to detect individual mRNA molecules. We constructed a doxycycline-controlled gene

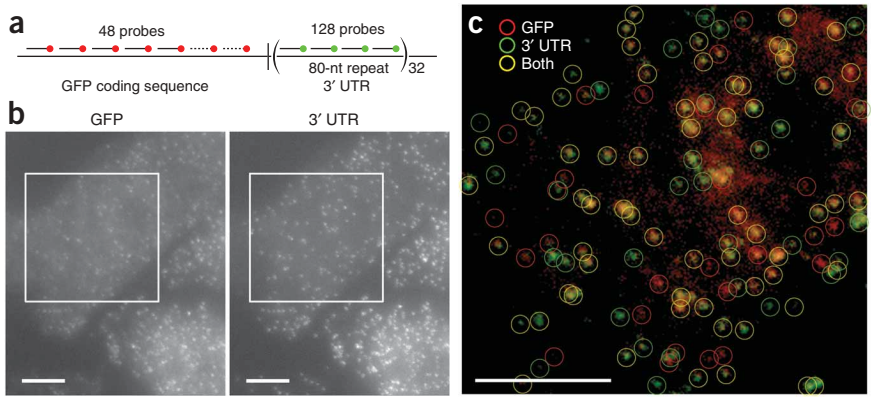

Figure 1 | Simultaneous detection of a unique sequence and a repeated sequence in individual mRNA molecules. (**a**) Schematic of the construct used. The 48 probes used to detect the GFP coding sequence were labeled with Alexa 594, and the four different probes used to detect the tandem repeat in the 3′ UTR were labeled with TMR. (**b**) Maximum intensity merges of a pair of *z*-image stacks of fluorescent images of CHO cells taken in the Alexa 594 channel (left) and the TMR channel (right). (**c**) False-color merge of the boxed regions in **b**, with circles representing computationally identified mRNA particles. Scale bars, 5 μm.

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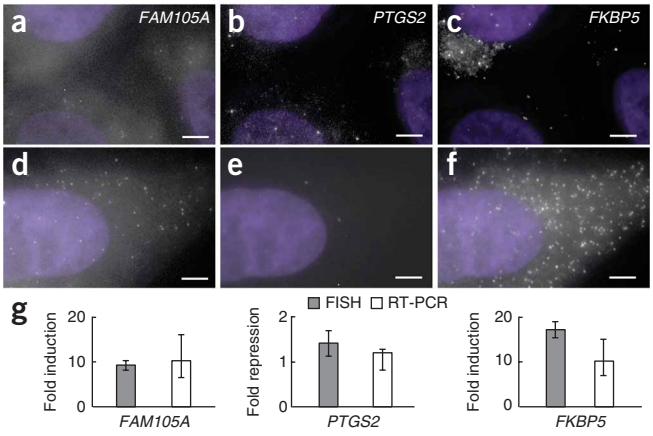

Figure 2 | Simultaneous imaging of three different mRNAs in mammalian cells. (**a–f**) Images showing fluorescent spots corresponding to *FAM105A*, *PTGS2* and *FKBP5* mRNAs in the same set of A549 cells not treated with dexamethasone (**a–c**) and in cells treated for 8 h with 24 nM dexamethasone (**d–f**). (**g**) Fold change in expression for all three genes as measured by FISH and real-time RT-PCR; error bars represent standard errors of measurements (see **Supplementary Methods**). All images are maximum merges of a *z*-stack of fluorescence images spanning the extent of the cells with nuclear 4,6-diamidino-2-phenylindole (DAPI) counterstaining in purple. Scale bars, 5 μm.

that produced an mRNA encoding GFP and contained 32 tandemly repeated 80-nucleotide-long sequences in its 3′ untranslated region (UTR); we then stably integrated this engineered gene into the genome of a Chinese hamster ovary cell line (**Fig. 1a**). Previously, we have shown that fluorescent probes targeted to tandemly repeated copies of probe-binding sequence results in FISH signals corresponding to individual molecules using a variety of methods, including a demonstration that the number of fluorescent spots per cell was about the same as the number of mRNA per cell, as measured by quantitative real-time reverse-transcriptase (RT)-PCR[10,11]. Here we targeted the coding region of the *GFP* mRNA with 48 different oligonucleotides labeled with Alexa 594 fluorophores and targeted each repeat sequence in the 3′ UTR with 4 oligonucleotides labeled with tetramethylrhodamine (TMR).

After hybridization, we imaged the cells with a pair of filter sets that could clearly distinguish between the two fluorophores. We found many 'particles' with a diameter of about 0.25 μm that appeared in both the TMR and Alexa 594 channels (**Fig. 1b**). The particles were identified computationally using an image processing program (**Supplementary Fig. 1**, **Supplementary Methods** and **Supplementary Software** online) that categorizes particles as being labeled with either the GFP-coding-sequence probes (TMR), the UTR-specific probes (Alexa-594) or both (**Fig. 1c**). Upon identifying and localizing particles in four fields of view similar to the ones shown in **Figure 1c**, we counted a total of 599 particles corresponding to GFP coding sequence–specific probes and 565 particles corresponding to the UTR-specific probes. Of these particles, 85% of the 'UTR particles' localized with the 'GFP particles', whereas 81% of the GFP particles colocalized with the UTR particles. The high degree of colocalization between particles detected by the previously established tandem-repeat detection method[10] and the particles detected via simultaneous probing with 48 different singly labeled oligonucleotides demonstrates the validity of using multiple single-labeled probes for the detection of endogenous transcripts. The fraction of particles that did not colocalize likely corresponded to mRNA molecules that lost either their coding sequence or their 3′ UTR via the natural processes of mRNA degradation. An analysis of fluorescence intensity of the colocalized spots showed that the spot intensities displayed a unimodal distribution (**Supplementary Fig. 2** online), arguing that the particles detected were individual molecules[10].

We also explored how the signal intensity varied with the number of probes by performing FISH using either the first 12, 24, or 36 probes or all 48 probes in our set. For this particular target mRNA, we found that particles could be detected with fewer probes, albeit with decreased intensity (**Supplementary Fig. 3a** online). However, our automatic spot-detection algorithm performed particularly well with 48 probes, detecting the same number of spots over a broad range of thresholds (**Supplementary Fig. 3b**). The number of probes required for robust signal is likely to depend on the target sequence, though, as accessibility to probes depends on the secondary structure in the RNA. Our method was at least as sensitive as the FISH-based method[6] described above (**Supplementary Fig. 4** online).

A potential use of our method is to simultaneously detect single molecules of multiple mRNAs in individual cells. To detect three different mRNAs at the same time, we designed probes specific for mRNAs encoding *FKBP5*, *PTGS2* and *FAM105A* in the human carcinoma cell line A549. We coupled these probes to the spectrally distinct fluorophores Cy5, Alexa 594 and TMR, respectively. Upon performing FISH with all three probes simultaneously, individual spots were visible in the three different fluorescence channels (**Fig. 2a–f**). The spots corresponding to different mRNAs did not overlap with each other. An intensity analysis showed that fluorescent spots did not bleed through into other channels (**Supplementary Fig. 5** online) and the use of an oxygen-scavenging mounting buffer ensured the stability of all fluorophores during the acquisition of image stacks (**Supplementary Fig. 6** online).

To demonstrate that our method of mRNA detection was specific and quantitative, we added to the growth medium a cell-permeant glucocorticoid, dexamethasone, which upregulates expression of *FKBP5* and *FAM105A*, and mildly downregulates expression of *PTGS2* in this cell line[12]. The mean number of *FKBP5* and *FAM105A* mRNAs measured by combining FISH with our spot-detection algorithm increased whereas the mean number of *PTGS2* mRNAs decreased (**Fig. 2a–f**). The values we obtained corresponded well to RT-PCR measurements of the fold induction and repression of these genes performed on the same samples, demonstrating that the fluorescent spots are the appropriate mRNAs and that we detected a majority of the mRNA molecules (**Fig. 2g**). This also demonstrated the effectiveness of our spot detection algorithm for accurate gene-expression quantification.

Our method also captured spatial information about the location of the mRNAs detected, a particularly important feature for studying development, in which mRNAs often display spatial patterning. We tested our method for efficacy in two commonly studied developmental systems: the nematode, *Caenorhabditis elegans*, and the fruit fly, *Drosophila melanogaster*. In the nematode, we constructed probes to detect mRNA molecules transcribed from the gene *elt-2*, a transcription factor that is expressed only in the nematode gut and only after the embryo has developed to the

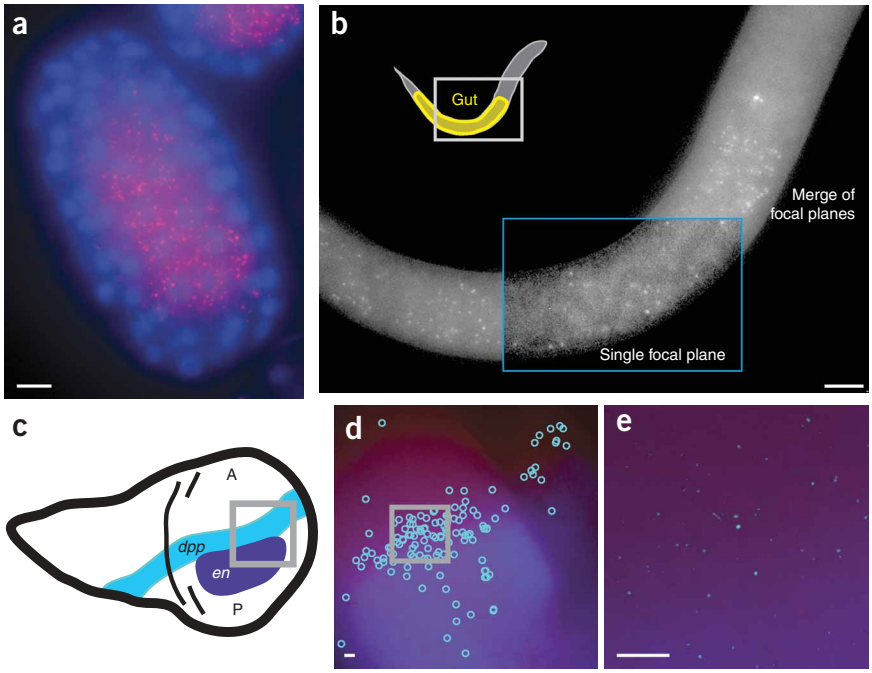

Figure 3 | Imaging localized mRNAs in *C. elegans* and *D. melanogaster*. (**a**) *elt-2* mRNA molecules (magenta) in an early stage *C. elegans* embryo (~100 cell stage); the nuclei were counterstained with DAPI (blue). (**b**) *elt-2* mRNA molecules in a *C. elegans* L1 larva. A single focal plane is shown in the boxed region, in which the intestinal track is visible. (**c**) A schematic of *dpp* and engrailed (*en*) expression in the imaginal wing discs of third instar *D. melanogaster* larvae. (**d**) Image showing the locations of the computationally identified *dpp* mRNA molecules (light blue circles) and Engrailed expression detected by immunofluorescence (dark blue). (**e**) Magnification of the boxed region in **d** showing enhanced *dpp* mRNA molecule signals (light blue) and Engrailed protein expression detected by immunofluorescence (dark blue). All images except the boxed portion in **b** are maximum merges of a *z*-stack of fluorescence images. Scale bars, 5 μm.

45-cell stage[13]. After hybridization of the probe set to both embryos and larvae, we found that *elt-2* mRNA molecules were present only in the gut region (**Fig. 3a**) of both the embryos and the larvae (**Fig. 3b**). Consistent with the known timing of the onset of expression[13], we only detected *elt-2* mRNAs in the gut of embryos older than the 45-cell stage.

In the fruit fly, one of the most well-studied examples of the localization of gene expression occurs in wing imaginal disc development[14]. The wing discs of fruit fly larvae display a remarkable set of gene expression patterns, one of which is the formation of a stripe of expression of the gene *dpp* in response to gradients of the morphogenic proteins Hedgehog and Engrailed[14] (**Fig. 3c**). To check whether this narrow stripe of *dpp* mRNA synthesis can be imaged, we constructed a set of singly labeled probes against *dpp* mRNA and performed FISH on imaginal wing discs isolated from third instar larvae while simultaneously performing immunofluorescence against Engrailed protein. We detected *dpp* mRNA in a stripe along the boundary of Engrailed protein expression (**Fig. 3d,e**), demonstrating both that the method can be used in wing imaginal discs and that the method can be easily combined with immunofluorescence detection.

Additional tests of our method showed that it was also applicable to *Saccharomyces cerevisae* and cultured rat hippocampal neurons, showing expected specificity in salt-induced expression of the *STL1* gene and dendritic localization of β-actin and *MAP2* mRNAs (**Supplementary Fig. 7** online).

Here we described a FISH method that allows for multiplex gene-expression profiling of transcripts across many model organisms. By using large numbers of singly labeled probes, our method generates uniform signals that can be computationally identified to yield accurate mRNA counts. In contrast, methods using heavily labeled probes (such as dendrimers) can suffer from false positives and negatives owing to individual probe misbinding or nonbinding events, respectively. Another advantage is the simplicity of probe generation and purification; by pooling, coupling and purifying the probes *en masse*, much of the complexity of probe preparation can be avoided. We created a web-based program for designing probe sets with optimally uniform G+C content (http://www.singlemoleculefish.com/). The simplicity of our method will likely facilitate genomic-scale studies of mRNA number and localization with applications in systems biology, cell biology, neurobiology and developmental biology.

Note: Supplementary information is available on the Nature Methods website.

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1. Kaufmann, B.B. & van Oudenaarden, A. *Curr. Opin. Genet. Dev.* **17**, 107–112 (2007).
2. St. Johnston, D. *Nat. Rev. Mol. Cell Biol.* **6**, 363–375 (2005).
3. Gall, J.G. *Proc. Natl. Acad. Sci. USA* **60**, 553–560 (1968).
4. Levsky, J.M. & Singer, R.H. *J. Cell Sci.* **116**, 2833–2838 (2003).
5. Raap, A.K. *et al. Hum. Mol. Genet.* **4**, 529–534 (1995).
6. Femino, A.M., Fay, F.S., Fogarty, K. & Singer, R.H. *Science* **280**, 585–590 (1998).
7. Maamar, H., Raj, A. & Dubnau, D. *Science* **317**, 526–529 (2007).
8. Femino, A.M., Fogarty, K., Lifshitz, L.M., Carrington, W. & Singer, R.H. *Methods Enzymol.* **361**, 245–304 (2003).
9. Randolph, J.B. & Waggoner, A.S. *Nucleic Acids Res.* **25**, 2923–2929 (1997).
10. Vargas, D.Y., Raj, A., Marras, S.A., Kramer, F.R. & Tyagi, S. *Proc. Natl. Acad. Sci. USA* **102**, 17008–17013 (2005).
11. Raj, A., Peskin, C.S., Tranchina, D., Vargas, D.Y. & Tyagi, S. *PLoS Biol.* **4**, e309 (2006).
12. Wang, J.C. *et al. Proc. Natl. Acad. Sci. USA* **101**, 15603–15608 (2004).
13. Fukushige, T., Hawkins, M.G. & McGhee, J.D. *Dev. Biol.* **198**, 286–302 (1998).
14. Sanicola, M., Sekelsky, J., Elson, S. & Gelbart, W.M. *Genetics* **139**, 745–756 (1995).

RNA *In Situ* Hybridization Using Digoxigenin-labeled cRNA Probes

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In recent years, the *in situ* hybridization (ISH) technique has found widespread application in both basic science and diagnostic clinical research. Its usefulness has been limited by the need for radioactive materials (short half-life of the radioisotope, long exposures for detection, and health and environmental hazards associated with the handling and disposal of the material; reference 1). Although radioactive probes are supposedly more sensitive than nonradioactive probes, the latter are more attractive because they are safe, shorten the length of the procedure, and provide optimum resolution (2).

The ISH technique has frequently been used to localize specific genes on metaphase chromosomes and to detect viral and bacterial genomes in infected tissues. The RNA *in situ* hybridization (RISH) technique for the examination of the mRNA expression has gained less attention due to the many technical problems associated with this technique.

In this technical report, we present a step-by-step protocol for a nonradioactive RISH technique on frozen sections using digoxigenin- (DIG-) labeled copy RNA (cRNA) probes. We demonstrate this technique on frozen sections of mouse kidney using DIG-labeled cRNA probes for the ectoenzyme aminopeptidase A, a low-copy RNA (3). For a high-copy RNA, we studied human psoriatic epidermis using a DIG-labeled cRNA probe for elafin/SKALP, an inhibitor of leukocyte elastase and proteinase 3 (reference 4). This protocol has been optimized to give strong hybridization signals, even with low-copy mRNA molecules. We have included hints at each step of this protocol to enhance the result of this often laborious and troublesome technique.

Materials and Methods

The cDNA clone coding for the mouse aminopeptidase A cDNA sequence was kindly provided by Dr. Max D. Cooper from the University of Alabama at Birmingham, USA (5). The cRNA probe for elafin/SKALP was kindly provided by Dr. J. Schalkwijk, department of Dermatology, University Hospital Nijmegen, The Netherlands.

Probe selection

The first step of choosing the type of probe (*i.e.*, DNA, RNA, or oligonucleotide) is essential for a good final result. For the optimization of the probe labeling, we have tested four methods of incorporating the DIG label:

- Direct labeling of cDNA molecules using the DIG DNA Labeling Kit (Genius 2 Kit)
- DIG labeling of oligonucleotides using the DIG Oligonucleotide Tailing Kit (Genius 6 Kit)
- DIG labeling of PCR products according to the method of Hannon *et al.* (6)
- DIG labeling of cRNA molecules using the DIG RNA Labeling Kit (Genius 4 Kit).

The RNA labeling method gave by far the best results. Therefore, we provide some hints on how to transcribe DIG-labeled cRNA molecules. For cRNA probe synthesis, one first has to subclone a cDNA molecule in an appropriate vector with flanking sequences that allow the insert to be transcribed (usually SP6 and T7 RNA polymerase sites flanking the multiple cloning sites of many commercially available vectors). One has to safeguard the length of the cDNA molecule since this seriously affects the hybridization efficiency. Usually, a cDNA length between 200 and 500 nucleotides gives the best results, allowing efficient hybridization and good penetration of the tissue. When it is known from Northern blotting experiments (Figure 1) that a molecule of interest is expressed as a high-copy RNA molecule but the target RNA is masked by proteins, it can be necessary to make probes with a length between 100 and 200 nucleotides for better penetration (Figure 2). After subcloning the cDNA molecule, the circular vector is linearized by restriction enzymes that cut the multiple cloning site in 2 orientations, allowing sense and antisense synthesis. This linearization step has to be controlled carefully by agarose gel electrophoresis, since circular molecules that are left in the digestion mixture affect the transcription efficiency. Subsequently, the linearized molecules are extracted from the digestion mixture.

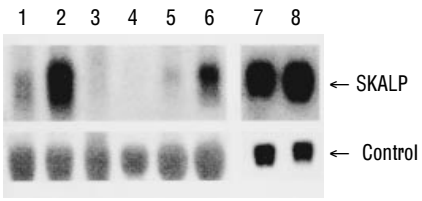

Figure 1 **Northern blot of SKALP mRNA in skin biopsies and cultured keratinocytes (upper panel).** 10 μg of total RNA was loaded. In biopsies from normal skin at 40 hours after injury (Lane 1), and in skin from psoriatic patients (Lane 2), a 0.8 kb message was found. Normal human epidermis was essentially negative (Lane 3) even on overloaded (50 μg RNA) gels. Keratinocytes cultured in KGM were essentially negative (Lane 4). Addition of 1% and 5% fetal calf serum (Lanes 5 and 6) induced SKALP mRNA in a dose-dependent fashion. Keratinocytes cultured on a 3T3 feeder layer give a strong signal (Lane 7) that is slightly increased when cells are cultured in suspension (Lane 8). The lower panel shows control hybridizations performed to check for equal RNA loading. In Lanes 1–6, a probe for 28S ribosomal RNA was used; in Lanes 7 and 8, a GAPDH probe was used. (reproduced with permission [4])

It is essential that these linearized molecules be purified by phenol extraction, since non-phenol-treated molecules gave a lower hybridization signal in the RISH. The incorporation of DIG-labeled nucleotides in these purified molecules was performed with the DIG RNA Labeling Kit (Genius 4 Kit) according to the instructions of Boehringer Mannheim. Nevertheless, we found two essential steps that, in our experiments, improve the labeling efficiency:

(i) Incubation of SP6 polymerase at 40°C instead of the recommended 37°C resulted in a more efficient labeling of cRNA probes.

(ii) The subsequent overnight precipitation step at –20°C after the labeling reaction results in more precipitated molecules than a 30 min precipitation at –70°C.

To control the labeling efficiency, we tested the labeled cRNA probes by limiting dilution and a spot blot test on nylon membranes using the DIG Luminescent Detection Kit (Genius 7 Kit), and by agarose gel electrophoresis to check for correct cRNA probe length. The sense and antisense cRNA probes should be labeled with equal efficiency compared to the controls provided in the kit. If the cRNA probes are not labeled with equal efficiency, the concentration of one cRNA

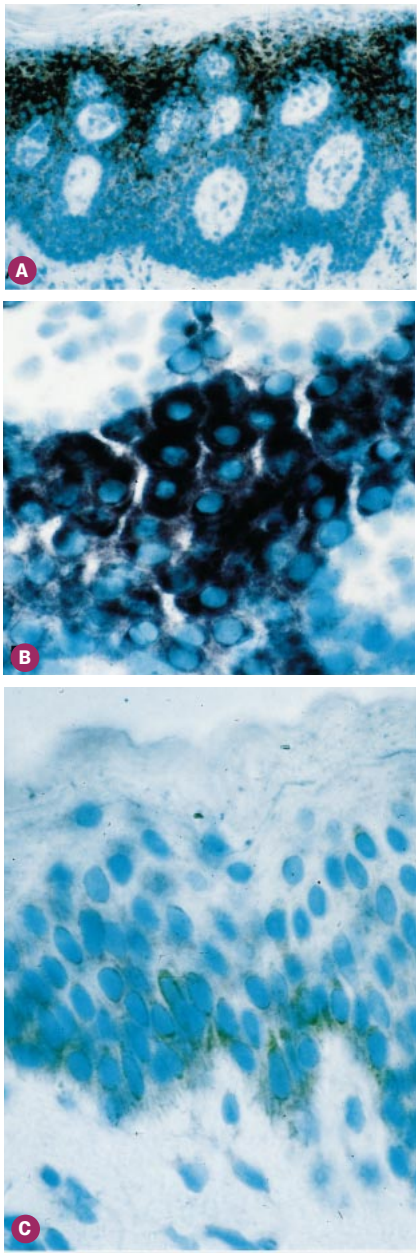

Figure 2 **RNA *in situ* hybridization on a frozen section of human psoriatic epidermis with DIG-labeled cRNA probes coding for human SKALP.** (A) an antisense cRNA probe (150 bp) was used on a 10 μm thick section (magnification x 125). (B) an antisense cRNA probe (150 bp) was used on a 20 μm thick section (magnification x500). (C) a sense cRNA probe (150 bp) was used on a 10 μm thick section (magnification x270). Intense staining of both the stratum spinosum and stratum granulosum is observed with the antisense probe, but not with the sense probe.

probe to another has to be adjusted in the RISH protocol. After the labeled cRNA probes have been tested and the correct concentrations determined, the cRNA probes should be aliquoted in polypropylene tubes at –70°C, since repeated thawing and freezing affects the stability of the labeled cRNA probe.

Tissues

When the objective of the RISH is the detection of low-copy RNA molecules, frozen sections are always preferred since paraffin-embedded material results in a loss of approximately 30% RNA and should only be used for the detection of high-abundance RNA. The method described here has been optimized for frozen sections. For the processing of tissue, clean all materials with 70% ethanol (70% EtOH) treated with diethyl pyrocarbonate (DEPC), and work as aseptically as possible. After removal, immediately snap-freeze tissue, and store it in liquid nitrogen. The time between removing and freezing the tissue should be kept to a minimum to avoid degradation of RNA (7).

Ten-micron-thick frozen sections should be cut with a 70% EtOH/DEPC-treated knife and directly mounted on Superfrost Plus slides (Menzel Gläser, Omnilabo, Breda, The Netherlands) to prevent detachment of the section during the procedure. For a higher specific signal, sections should be thicker than 10 μm, whereas sections thinner than 10 μm should be used when a better localization is needed. Several slides and coatings have been tested, but the best results have been obtained with Superfrost Plus slides.

Pretreatment

Directly after mounting on Superfrost plus slides, the sections are heated on a heating stove (see the protocol in Table 1) to fix the RNA in the tissue. The time and temperature varies from 10–120 seconds and from 50–90°C, depending on the tissue and type of RNA. Test this heat fixation for each RNA molecule of interest. The sections are subsequently dried for 30 minutes to prevent them from repelling delipidizing liquids.

In many protocols, proteinase K treatment has been included to enhance the penetration of the probes. We found that this proteinase K treatment seriously affected the morphology of the section but did slightly improve the signal/noise ratio on a 4% paraformaldehyde-fixed section, and therefore should not be used in this protocol.

We found that tissues from mice frequently had lipid vesicles on top of the sections in which the developing substrate was trapped, causing high nonspecific backgrounds (Figure 3). To remove these lipid vesicles, we dehydrated the sections with ethanol, which led to a reduction of the background, but also to a reduction of the signal, presumably caused by extraction of RNA from the section (Figure 4). It is argued that this RNA extraction during ethanol treatment can be reduced by adding ammonium acetate. In our hands, however, we also found substantial reduction of the specific signal when we added ammonium sulfate to the ethanol. On the other hand, delipidization of the sections with chloroform also led to reduction of the background, but did not affect the signal (Figure 5). As a control, the binding of the sense cRNA probe is shown in Figure 6. This chloroform treatment is followed by drying of the section to evaporate the

Pretreatment		
1x	2 min	Heat fixation at 50°C
1x	30 min	Drying sections
1x	5 min	Chloroform (optional)
1x	7 min	4% paraformaldehyde/PBS
1x	3 min	PBS
2x	5 min	2x SSC (1x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2)
Prehybridization		
1x	60 min	100 μl Prehybridization buffer (4x SSC, 10% dextran sulfate, 1x Denhardt's solution [0.02% Ficoll® 400, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin], 2 mM EDTA, 50% deionized formamide, 500 μg/ml herring sperm DNA) at 37°–50°C
Hybridization		
1x	16 hours	100 μl Prehybridization buffer + 200 ng/ml DIG-labeled cRNA probe at 37°–50°C (37°C is standard)
Posthybridization		
1x	5 min	2x SSC at 37°C
3x	5 min	60% formamide/0.2x SSC at 37°C
2x	5 min	2x SSC
Immunological detection		
1x	5 min	100 mM Tris-HCl (pH 7.5), 150 mM NaCl
1x	30 min	100 mM Tris-HCl (pH 7.5), 150 mM NaCl; saturated with blocking mix
1x	120 min	1:200 polyclonal sheep anti-digoxigenin-Fab fragments conjugated to alkaline phosphatase (750 U/ml) in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl; saturated with blocking mix
2x	5 min	100 mM Tris-HCl (pH 7.5), 150 mM NaCl
1x	10 min	100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$
1x	16 hours	0.18 mg/ml BCIP and 0.34 mg/ml NBT in buffer 3 + 240 μg/ml levamisole
1x	5 min	10 mM Tris (pH 8), 1 mM EDTA
Counterstain		
1x	5 min	dH_2O
1x	5-10 min	methylene green at 37°C
1x	5 min	dH_2O
		Mount coverslips in Kaiser's solution

Table 1. RNA *in situ* hybridization protocol using digoxigenin-labeled cRNA probes. All solutions are prepared with 1% DEPC-treated water. All procedures are performed at room temperature unless stated otherwise.

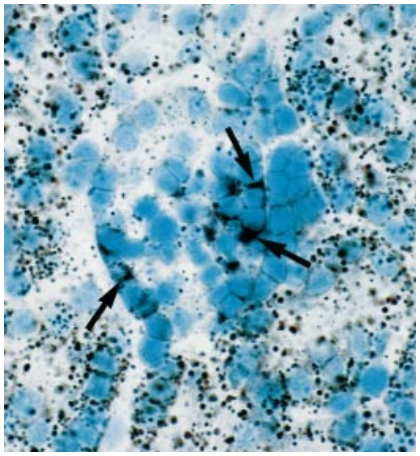

Figure 3 **RNA *in situ* hybridization on a 10 μm thick frozen kidney section of a male BALB/c mouse with a digoxigenin-labeled cRNA probe coding for mouse aminopeptidase A. Note the many nonspecific lipid vesicles in the section. The specific hybridization signal is seen in the cells of the glomerulus (arrows; magnification x600).**

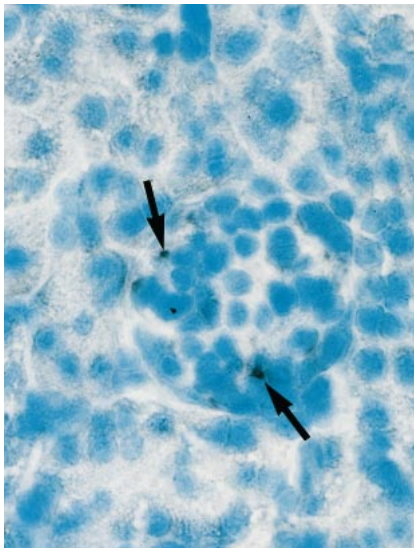

Figure 4 **Effect of tissue pretreatment with ethanol on hybridization signals.** RNA *in situ* hybridization was performed on a 10 μm thick frozen kidney section of the same BALB/c mouse shown in Figure 3 with a digoxigenin-labeled cRNA probe coding for mouse aminopeptidase A. Pretreatment of the sections with ethanol prior to the hybridization diminished the background (nonspecific signals in the lipid vesicles), but unfortunately also the hybridization signal (arrows; magnification x600).

chloroform. This chloroform treatment is optional and has been included in the protocol (Table 1), but should be omitted at first in pilot studies. The tissue is subsequently fixed in 4% paraformaldehyde to obtain a satisfying morphology and retention of the RNA in the section.

Prehybridization and hybridization

The composition of the hybridization buffer is very important for good hybridization of the cRNA molecule and the target RNA molecule. Researchers have employed many hybridization buffers containing more or less the same components in varying concentrations that depend on the melting temperature of the cRNA molecule. The hybridization buffer in the accompanying protocol (Table 1) contains some essential components for good hybridization. Denhardt's solution is added as a blocking reagent to prevent nonspecific binding of the cRNA probe to the section. The denatured herring sperm DNA is added as a carrier molecule for the cRNA probe to improve the hybridization. The Ca^{2+}-chelating agent EDTA is added to minimize the destabilizing effects of Ca^{2+} on the hybridization of the cRNA molecule to its target. Dextran sulfate, finally, is added to improve the contact between the cRNA molecule and the target RNA molecule.

First, the sections are prehybridized to block nonspecific targets to which the cRNA probe might "stick" during hybridization. This hybridization buffer is subsequently replaced by the labeled cRNA probe, at an optimal concentration of 200 ng/ml, in the same hybridization buffer. This concentration is used when the cRNA probe is labeled with equal efficiency as the controls from the DIG RNA Labeling Kit (Genius 4 Kit). If the cRNA probe is not efficiently labeled, the concentration of the cRNA probe should be adjusted. We did not use coverslips during the hybridization since this decreased the signal up to four times. To increase the hybridization efficiency and to prevent smudging of the substrate, we circled the sections with a silicone pen (Dako A/S, Glostrup, Denmark).

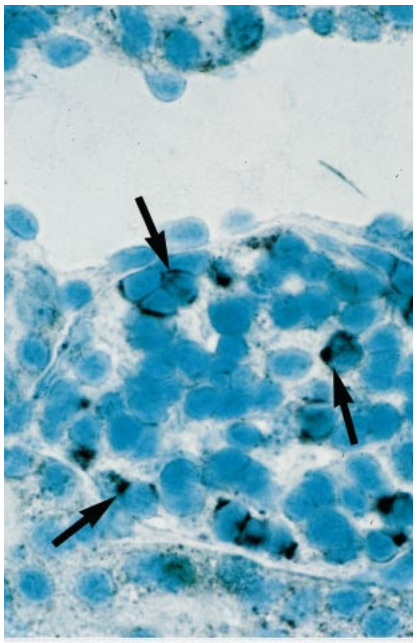

Figure 5 **Effect of tissue pretreatment with chloroform on hybridization signals.** RNA *in situ* hybridization was performed on a 10 μm thick frozen kidney section of the same BALB/c mouse as shown in Figures 3 and 4 with a digoxigenin-labeled antisense cRNA probe coding for mouse aminopeptidase A. Pretreatment of the section with chloroform prior to the hybridization delipidized the section with concomitant disappearance of the nonspecific signal in the lipid vesicles. The specific hybridization signal was undiminished (arrows; magnification x600).

Posthybridization

After the hybridization, wash unbound cRNA probe from the section. When the salt concentration in the washing buffer is lowered, the washing step will be more stringent, and nonspecifically bound cRNA probe will be washed away. The washing step should be performed at a temperature 5°C beneath the melting temperature of the probe. Ethanol treatment of the section after hybridization has reportedly improved the signal-to-noise ratio (8), but we found much lower signals during ethanol treatment. RNase digestion after the hybridization can be used to digest single-stranded RNA molecules and to improve the signal-to-noise ratio; use an RNase with no or very low nicking activity to avoid digestion of the bound cRNA probe.

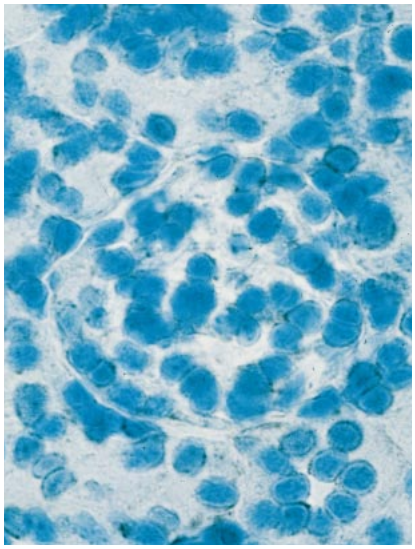

Figure 6 **RNA *in situ* hybridization on a 10 μm thick frozen kidney section of the same BALB/c mouse as shown in Figures 3–5 with a digoxigenin-labeled sense cRNA probe coding for mouse aminopeptidase A.** The section was pretreated with chloroform prior to the hybridization, and no signal could be seen, indicating the specificity of the antisense hybridization signal (magnification x600).

Immunological detection

For the immunological detection of the DIG label, we followed the instructions of the manufacturer. We did not, however, use the 1x blocking mix, but rather 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, saturated with milk powder. The development of the alkaline phosphatase-conjugated anti-DIG antibody with the NBT/BCIP substrate can go on indefinitely, but the time to obtain an excellent signal/ noise ratio is dependent on the abundance of the RNA molecules of interest. For the detection of low-abundance RNA, we develop overnight. This development should be carried out with the slides standing up in a small container to prevent nonspecifically converted substrate from falling on the section. Endogenous alkaline phosphatase that might convert the substrate can be inhibited with levamisole (see Table 1).

Counterstain

We counterstain the nuclei with methylene green to obtain a good resolution and a good contrast between the specific signal (colored purple to black) and the surrounding tissue. We mount the slides in an aqueous mounting solution (Kaiser's), since other mounting solutions induce the formation of substrate crystals on the section. These crystals are formed spontaneously in the NBT and BCIP stocks during prolonged storage; centrifuging the stocks and subsequently filtering the NBT/BCIP substrate through a 0.25 µm filter prior to use diminishes these crystals.

Conclusion

We have provided a standard protocol for RISH on low- and high-copy RNA molecules and provided some hints at each step. Using this protocol, one should be able to perform RISH on each RNA molecule of interest, with only minor modifications depending on the abundance of the RNA of interest. ■

Product	Cat. No.	Size
DIG RNA Labeling Kit (Genius 4 Kit)	1175 025	1 kit (2 x 10 labeling reactions)
DIG DNA Labeling and Detection Kit (Genius 1 Kit)	1093 657	1 kit (25 labeling and 50 detection [100 cm^2] reactions)
DIG Oligonucleotide Tailing Kit (Genius 6 Kit)	1417 231	1 kit (25 tailing reactions, for 100 pmol oligonucleotides

Also Available	Cat. No.	Size
PCR DIG Probe Synthesis Kit*	1636 090	1 kit (25 labeling reactions)
DIG Wash and Block Buffer Set	1585 762	1 set (at least 30 blots, 100 cm^2)

See page 17 for local pricing.

*Purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for molecular biology research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, *i.e.*, an authorized thermal cycler.

References

1. Moorman, A. F. M., de Boer, P. A. J., Vermeulen, J. L. M. and Lamers, W. H. (1993) Practical aspects of radioisotopic *in situ* hybridization on RNA. *Histochem J.* **25**:251.
2. Birk, P. E. and Grimm, P. C. (1994) Rapid Nonradioactive *in situ* Hybridization for Interleukin-2 mRNA with Riboprobes Generated Using the Polymerase Chain Reaction. *Journal of Immunological Methods* **167**:83.
3. Assmann, K. J. M., van Son, J. P. H. F., Dijkman, H. B. P. M and Koene, R. A. P. (1992) A nephritogenic rat monoclonal antibody to mouse aminopeptidase A. Induction of massive albuminuria after a single intravenous injection. *J. Exp. Med.* **175**:623.
4. Alkemade, J. A. C., Molhuizen, H. O. F., Ponec, M., Kempenaar, J. A., Zeeuwen, P .L. J. M., de Jongh, G. J., van Vlijmen-Willems, I. M. J. J., van Erp, P. E. J., van de Kerkhof, P. C. M. and Schalkwijk, J. (1994) SKALP/elafin is an inducible proteinase inhibitor in human epidermal keratinocytes. *J. Cell Sci.* **107**:2335.
5. Wu, Q., Lahti, J. M., Air, G. M., Burrows, P. D. and Cooper, M. D. (1990) Molecular cloning of the murine BP-1/6C3 antigen: a member of the zinc-dependent metallopeptidase family. *Proc. Natl. Acad. Sci. USA* **87**:993.
6. Hannon, K., Johnstone, E., Craft, L. S., Little, S. P., Smith, C. K., Heiman, M. L. and Santerre, R. F. (1993) Synthesis of PCR-derived, single-stranded DNA probes suitable for *in situ* hybridization. *Anal. Biochem.* **212**:421.
7. Barton, A. J. L., Pearson, R. C. A., Najlerahim, A. and Harrison, P. J. (1993): Pre- and postmortem influences on brain RNA. *J. Neurochem.* **61**:1.
8. Yun, K. and de Smet, K. A. L. (1993) Detection of insulin mRNA by *in situ* hybridization and northern blot hybridization using isotopic and non-isotopic probes. *Acta Histochem. Cytochem.* **26**(3):189.