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United States Patent**8,535,889****Larson , et al.****September 17, 2013*****Digital analyte analysis*****Abstract**

The invention generally relates to droplet based digital PCR and methods for analyzing a target nucleic acid using the same. In certain embodiments, methods of the invention involve forming sample droplets containing, on average, a single target nucleic acid, amplifying the target in the droplets, excluding droplets containing amplicon from the target and amplicon from a variant of the target, and analyzing target amplicons.

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References Cited [Referenced By]

U.S. Patent Documents

<u>2097692</u>	November 1937	Fiegel
<u>2164172</u>	June 1939	Dalton
<u>2656508</u>	October 1953	Coulter
<u>2692800</u>	October 1954	Nichols et al.
<u>2797149</u>	June 1957	Skeggs
<u>2879141</u>	March 1959	Skeggs
<u>2971700</u>	February 1961	Peeps
<u>3479141</u>	November 1969	Smythe et al.
<u>3608821</u>	September 1971	Simm et al.
<u>3698635</u>	October 1972	Sickles
<u>3816331</u>	June 1974	Brown, Jr. et al.
<u>3930061</u>	December 1975	Scharfenberger
<u>3960187</u>	June 1976	Stock et al.
<u>3980541</u>	September 1976	Aine
<u>3982541</u>	September 1976	L'Esperance, Jr.
<u>4014469</u>	March 1977	Sato
<u>4022575</u>	May 1977	Hansen et al.
<u>4034966</u>	July 1977	Suh et al.
<u>4059552</u>	November 1977	Zweigle et al.
<u>4091042</u>	May 1978	Alexanderson et al.
<u>4117550</u>	September 1978	Folland et al.
<u>4130394</u>	December 1978	Negersmith
<u>4210809</u>	July 1980	Pelavin

<u>4253846</u>	March 1981	Smythe et al.
<u>4266721</u>	May 1981	Sickles
<u>4279345</u>	July 1981	Allred
<u>4297345</u>	October 1981	Howarth
<u>4315754</u>	February 1982	Ruzicka et al.
<u>4378957</u>	April 1983	Malkin et al.
<u>4383767</u>	May 1983	Jido
<u>4439980</u>	April 1984	Biblarz et al.
<u>4508265</u>	April 1985	Jido
<u>4533634</u>	August 1985	Maldonado et al.
<u>4585209</u>	April 1986	Aine et al.
<u>4618476</u>	October 1986	Columbus
<u>4675285</u>	June 1987	Clark et al.
<u>4676274</u>	June 1987	Brown
<u>4683195</u>	July 1987	Mullis et al.
<u>4683202</u>	July 1987	Mullis
<u>4739044</u>	April 1988	Stabinsky
<u>4757141</u>	July 1988	Fung et al.
<u>4767515</u>	August 1988	Scott et al.
<u>4767929</u>	August 1988	Valentine
<u>4779805</u>	October 1988	Jackson et al.
<u>4801086</u>	January 1989	Noakes
<u>4801529</u>	January 1989	Perlman
<u>4829996</u>	May 1989	Noakes et al.
<u>4853336</u>	August 1989	Saros et al.
<u>4865444</u>	September 1989	Green et al.
<u>4883750</u>	November 1989	Whiteley et al.
<u>4908112</u>	March 1990	Pace
<u>4931225</u>	June 1990	Cheng
<u>4941959</u>	July 1990	Scott
<u>4962885</u>	October 1990	Coffee
<u>4963498</u>	October 1990	Hillman et al.
<u>4981580</u>	January 1991	Auer
<u>4996004</u>	February 1991	Bucheler et al.
<u>5091652</u>	February 1992	Mathies et al.
<u>5096615</u>	March 1992	Prescott et al.
<u>5122360</u>	June 1992	Harris et al.
<u>5180662</u>	January 1993	Sitkovsky

<u>5185099</u>	February 1993	Delpuech et al.
<u>5188290</u>	February 1993	Gebauer et al.
<u>5188291</u>	February 1993	Cross
<u>5204112</u>	April 1993	Hope et al.
<u>5207973</u>	May 1993	Harris et al.
<u>5241159</u>	August 1993	Chatterjee et al.
<u>5260466</u>	November 1993	McGibbon
<u>5262027</u>	November 1993	Scott
<u>5270163</u>	December 1993	Gold et al.
<u>5296375</u>	March 1994	Kricka et al.
<u>5304487</u>	April 1994	Wilding et al.
<u>5310653</u>	May 1994	Hanausek-Walaszek et al.
<u>5313009</u>	May 1994	Guenkel et al.
<u>5344594</u>	September 1994	Sheridon
<u>5378957</u>	January 1995	Kelly
<u>5397605</u>	March 1995	Barbieri et al.
<u>5399461</u>	March 1995	Van et al.
<u>5399491</u>	March 1995	Kacian et al.
<u>5403617</u>	April 1995	Haaland
<u>5413924</u>	May 1995	Kosak et al.
<u>5417235</u>	May 1995	Wise et al.
<u>5427946</u>	June 1995	Kricka et al.
<u>5445934</u>	August 1995	Fodor et al.
<u>5452878</u>	September 1995	Gravesen et al.
<u>5452955</u>	September 1995	Lundstrom
<u>5454472</u>	October 1995	Benecke et al.
<u>5460945</u>	October 1995	Springer et al.
<u>5475096</u>	December 1995	Gold et al.
<u>5480614</u>	January 1996	Kamahori
<u>5486335</u>	January 1996	Wilding et al.
<u>5498392</u>	March 1996	Wilding et al.
<u>5500415</u>	March 1996	Dollat et al.
<u>5503851</u>	April 1996	Mank et al.
<u>5512131</u>	April 1996	Kumar et al.
<u>5516635</u>	May 1996	Ekins et al.
<u>5518709</u>	May 1996	Sutton et al.
<u>5523162</u>	June 1996	Franz et al.
<u>5587128</u>	December 1996	Wilding et al.

<u>5604097</u>	February 1997	Brenner
<u>5612188</u>	March 1997	Shuler et al.
<u>5616478</u>	April 1997	Chetverin et al.
<u>5617997</u>	April 1997	Kobayashi et al.
<u>5635358</u>	June 1997	Wilding et al.
<u>5636400</u>	June 1997	Young
<u>5641658</u>	June 1997	Adams et al.
<u>5643729</u>	July 1997	Taniguchi et al.
<u>5655517</u>	August 1997	Coffee
<u>5656155</u>	August 1997	Norcross et al.
<u>5661222</u>	August 1997	Hare
<u>5662874</u>	September 1997	David
<u>5670325</u>	September 1997	Lapidus et al.
<u>5681600</u>	October 1997	Antinone et al.
<u>5695934</u>	December 1997	Brenner
<u>5726026</u>	March 1998	Wilding et al.
<u>5726404</u>	March 1998	Brody
<u>5733526</u>	March 1998	Trevino et al.
<u>5739036</u>	April 1998	Parris
<u>5744366</u>	April 1998	Kricka et al.
<u>5750988</u>	May 1998	Apffel et al.
<u>5762775</u>	June 1998	DePaoli et al.
<u>5779868</u>	July 1998	Parce et al.
<u>5783431</u>	July 1998	Peterson et al.
<u>5840506</u>	November 1998	Giordano
<u>5846719</u>	December 1998	Brenner et al.
<u>5849491</u>	December 1998	Radomski et al.
<u>5858187</u>	January 1999	Ramsey et al.
<u>5858655</u>	January 1999	Arnold
<u>5858670</u>	January 1999	Lam et al.
<u>5863722</u>	January 1999	Brenner
<u>5868322</u>	February 1999	Loucks, Jr. et al.
<u>5872010</u>	February 1999	Karger et al.
<u>5876771</u>	March 1999	Sizer et al.
<u>5880071</u>	March 1999	Parce et al.
<u>5882680</u>	March 1999	Suzuki et al.
<u>5884846</u>	March 1999	Tan

<u>5887755</u>	March 1999	Hood, III
<u>5888746</u>	March 1999	Tabiti et al.
<u>5888778</u>	March 1999	Shuber
<u>5904933</u>	May 1999	Riess et al.
<u>5921678</u>	July 1999	Desai et al.
<u>5927852</u>	July 1999	Serafin
<u>5928870</u>	July 1999	Lapidus et al.
<u>5932100</u>	August 1999	Yager et al.
<u>5935331</u>	August 1999	Naka et al.
<u>5942056</u>	August 1999	Singh
<u>5942443</u>	August 1999	Parce et al.
<u>5958203</u>	September 1999	Parce et al.
<u>5972187</u>	October 1999	Parce et al.
<u>5980936</u>	November 1999	Krafft et al.
<u>5989815</u>	November 1999	Skolnick et al.
<u>5989892</u>	November 1999	Nishimaki et al.
<u>5995341</u>	November 1999	Tanaka et al.
<u>5997636</u>	December 1999	Gamarnik et al.
<u>6008003</u>	December 1999	Haak-Frendscho et al.
<u>6023540</u>	February 2000	Walt et al.
<u>6028066</u>	February 2000	Unger
<u>6042709</u>	March 2000	Parce et al.
<u>6045755</u>	April 2000	Lebl et al.
<u>6046056</u>	April 2000	Parce et al.
<u>6048551</u>	April 2000	Hilffinger et al.
<u>6068199</u>	May 2000	Coffee
<u>6080295</u>	June 2000	Parce et al.
<u>6086740</u>	July 2000	Kennedy
<u>6096495</u>	August 2000	Kasai et al.
<u>6103537</u>	August 2000	Ullman et al.
<u>6105571</u>	August 2000	Coffee
<u>6105877</u>	August 2000	Coffee
<u>6116516</u>	September 2000	Ganan-Calvo
<u>6118849</u>	September 2000	Tanimori et al.
<u>6119953</u>	September 2000	Ganan-Calvo et al.
<u>6120666</u>	September 2000	Jacobson et al.
<u>6124388</u>	September 2000	Takai et al.
<u>6124439</u>	September 2000	Friedman et al.

<u>6130052</u>	October 2000	Van Baren et al.
<u>6130098</u>	October 2000	Handique et al.
<u>6137214</u>	October 2000	Raina
<u>6138077</u>	October 2000	Brenner
<u>6139303</u>	October 2000	Reed et al.
<u>6140053</u>	October 2000	Koster
<u>6143496</u>	November 2000	Brown et al.
<u>6149789</u>	November 2000	Benecke et al.
<u>6150180</u>	November 2000	Parce et al.
<u>6150516</u>	November 2000	Brenner et al.
<u>6165778</u>	December 2000	Kedar
<u>6171796</u>	January 2001	An et al.
<u>6171850</u>	January 2001	Nagle et al.
<u>6172214</u>	January 2001	Brenner
<u>6172218</u>	January 2001	Brenner
<u>6174160</u>	January 2001	Lee et al.
<u>6174469</u>	January 2001	Ganan-Calvo
<u>6180372</u>	January 2001	Franzen
<u>6184012</u>	February 2001	Neri et al.
<u>6187214</u>	February 2001	Ganan-Calvo
<u>6189803</u>	February 2001	Ganan-Calvo
<u>6196525</u>	March 2001	Ganan-Calvo
<u>6197335</u>	March 2001	Sherman
<u>6197835</u>	March 2001	Ganan-Calvo
<u>6203993</u>	March 2001	Shuber et al.
<u>6210396</u>	April 2001	MacDonald et al.
<u>6210891</u>	April 2001	Nyren et al.
<u>6210896</u>	April 2001	Chan
<u>6214558</u>	April 2001	Shuber et al.
<u>6221654</u>	April 2001	Quake et al.
<u>6227466</u>	May 2001	Hartman et al.
<u>6234402</u>	May 2001	Ganan-Calvo
<u>6235383</u>	May 2001	Hong et al.
<u>6235475</u>	May 2001	Brenner et al.
<u>6241159</u>	June 2001	Ganan-Calvo et al.
<u>6243373</u>	June 2001	Turock
<u>6248378</u>	June 2001	Ganan-Calvo
<u>6251661</u>	June 2001	Urabe et al.

<u>6252129</u>	June 2001	Coffee
<u>6258568</u>	July 2001	Nyren
<u>6258858</u>	July 2001	Nakajima et al.
<u>6263222</u>	July 2001	Diab et al.
<u>6266459</u>	July 2001	Walt et al.
<u>6267353</u>	July 2001	Friedline et al.
<u>6267858</u>	July 2001	Parce et al.
<u>6268165</u>	July 2001	O'Brien
<u>6268222</u>	July 2001	Chandler et al.
<u>6274320</u>	August 2001	Rothberg et al.
<u>6274337</u>	August 2001	Parce et al.
<u>6294344</u>	September 2001	O'Brien
<u>6296673</u>	October 2001	Santarsiero et al.
<u>6299145</u>	October 2001	Ganan-Calvo
<u>6301055</u>	October 2001	Legrand et al.
<u>6306659</u>	October 2001	Parce et al.
<u>6310354</u>	October 2001	Hanninen et al.
<u>6310653</u>	October 2001	Malcolm, Jr. et al.
<u>6316208</u>	November 2001	Roberts et al.
<u>6316213</u>	November 2001	O'Brien
<u>6318640</u>	November 2001	Coffee
<u>6336463</u>	January 2002	Ohta
<u>6344325</u>	February 2002	Quake et al.
<u>6352828</u>	March 2002	Brenner
<u>6355193</u>	March 2002	Stott
<u>6355198</u>	March 2002	Kim et al.
<u>6357670</u>	March 2002	Ganan-Calvo
<u>6386463</u>	May 2002	Ganan-Calvo
<u>6391559</u>	May 2002	Brown et al.
<u>6394429</u>	May 2002	Ganan-Calvo
<u>6399339</u>	June 2002	Wolberg et al.
<u>6399389</u>	June 2002	Parce et al.
<u>6403373</u>	June 2002	Scanlan et al.
<u>6405936</u>	June 2002	Ganan-Calvo
<u>6408878</u>	June 2002	Unger et al.
<u>6409832</u>	June 2002	Weigl et al.
<u>6429025</u>	August 2002	Parce et al.
<u>6429148</u>	August 2002	Chu et al.

<u>6432143</u>	August 2002	Kubiak et al.
<u>6432148</u>	August 2002	Ganan-Calvo
<u>6432630</u>	August 2002	Blankenstein
<u>6439103</u>	August 2002	Miller
<u>6440706</u>	August 2002	Vogelstein et al.
<u>6450139</u>	September 2002	Watanabe
<u>6450189</u>	September 2002	Ganan-Calvo
<u>6454193</u>	September 2002	Busick et al.
<u>6464336</u>	October 2002	Sharma
<u>6464886</u>	October 2002	Ganan-Calvo
<u>6475441</u>	November 2002	Parce et al.
<u>6481648</u>	November 2002	Zimmermann
<u>6489103</u>	December 2002	Griffiths et al.
<u>6503933</u>	January 2003	Moloney et al.
<u>6506609</u>	January 2003	Wada et al.
<u>6508988</u>	January 2003	Van Dam et al.
<u>6520425</u>	February 2003	Reneker
<u>6524456</u>	February 2003	Ramsey et al.
<u>6540395</u>	April 2003	Muhlbauer et al.
<u>6540895</u>	April 2003	Spence et al.
<u>6551836</u>	April 2003	Chow et al.
<u>6553944</u>	April 2003	Allen et al.
<u>6553960</u>	April 2003	Yoshikawa et al.
<u>6554202</u>	April 2003	Ganan-Calvo
<u>6557334</u>	May 2003	Jager
<u>6557834</u>	May 2003	Ganan-Calvo
<u>6558944</u>	May 2003	Parce et al.
<u>6558960</u>	May 2003	Parce et al.
<u>6560030</u>	May 2003	Legrand et al.
<u>6565010</u>	May 2003	Anderson et al.
<u>6569631</u>	May 2003	Pantoliano et al.
<u>6576420</u>	June 2003	Carson et al.
<u>6591852</u>	July 2003	McNeely et al.
<u>6592321</u>	July 2003	Bonker et al.
<u>6592821</u>	July 2003	Wada et al.
<u>6608726</u>	August 2003	Legrand et al.
<u>6610499</u>	August 2003	Fulwyler et al.

<u>6614598</u>	September 2003	Quake et al.
<u>6627603</u>	September 2003	Bibette et al.
<u>6630006</u>	October 2003	Santarsiero et al.
<u>6630353</u>	October 2003	Parce et al.
<u>6632619</u>	October 2003	Harrison et al.
<u>6638749</u>	October 2003	Beckman et al.
<u>6645432</u>	November 2003	Anderson et al.
<u>6646253</u>	November 2003	Rohwer et al.
<u>6653626</u>	November 2003	Fischer et al.
<u>6656267</u>	December 2003	Newman
<u>6659370</u>	December 2003	Inoue
<u>6660252</u>	December 2003	Matathia et al.
<u>6670142</u>	December 2003	Lau et al.
<u>6679441</u>	January 2004	Borra et al.
<u>6680178</u>	January 2004	Harris et al.
<u>6682890</u>	January 2004	Mack et al.
<u>6717136</u>	April 2004	Andersson et al.
<u>6729561</u>	May 2004	Hirae et al.
<u>6739036</u>	May 2004	Koike et al.
<u>6744046</u>	June 2004	Valaskovic et al.
<u>6752922</u>	June 2004	Huang et al.
<u>6753147</u>	June 2004	Vogelstein et al.
<u>6766817</u>	July 2004	da Silva
<u>6767194</u>	July 2004	Jeon et al.
<u>6767704</u>	July 2004	Waldman et al.
<u>6790328</u>	September 2004	Jacobson et al.
<u>6793753</u>	September 2004	Unger et al.
<u>6797056</u>	September 2004	David
<u>6800849</u>	October 2004	Staats
<u>6806058</u>	October 2004	Jespersion et al.
<u>6808382</u>	October 2004	Lanfranchi
<u>6808882</u>	October 2004	Griffiths et al.
<u>6814980</u>	November 2004	Levy et al.
<u>6818395</u>	November 2004	Quake et al.
<u>6832787</u>	December 2004	Renzi
<u>6833242</u>	December 2004	Quake et al.
<u>6841350</u>	January 2005	Ogden et al.

<u>6872250</u>	March 2005	David et al.
<u>6890487</u>	May 2005	Sklar et al.
<u>6897018</u>	May 2005	Yuan et al.
<u>6905844</u>	June 2005	Kim
<u>6918404</u>	July 2005	Dias da Silva
<u>6926313</u>	August 2005	Renzi
<u>6935768</u>	August 2005	Lowe et al.
<u>6936417</u>	August 2005	Orntoft
<u>6942978</u>	September 2005	O'Brien
<u>6949342</u>	September 2005	Golub et al.
<u>6960437</u>	November 2005	Enzelberger et al.
<u>6974667</u>	December 2005	Horne et al.
<u>6998232</u>	February 2006	Feinstein et al.
<u>7022472</u>	April 2006	Robbins et al.
<u>7041481</u>	May 2006	Anderson et al.
<u>7049072</u>	May 2006	Seshi
<u>7056674</u>	June 2006	Baker et al.
<u>7057026</u>	June 2006	Barnes et al.
<u>7066586</u>	June 2006	da Silva
<u>7068874</u>	June 2006	Wang et al.
<u>7078180</u>	July 2006	Genetta
<u>7081192</u>	July 2006	Wang et al.
<u>7081340</u>	July 2006	Baker et al.
<u>7090983</u>	August 2006	Muramatsu et al.
<u>7115230</u>	October 2006	Sundararajan et al.
<u>7118910</u>	October 2006	Unger et al.
<u>7129091</u>	October 2006	Ismagilov et al.
<u>7138233</u>	November 2006	Griffiths et al.
<u>7153700</u>	December 2006	Pardee et al.
<u>7156917</u>	January 2007	Moriyama et al.
<u>7163801</u>	January 2007	Reed
<u>7169560</u>	January 2007	Lapidus et al.
<u>7171311</u>	January 2007	Dai et al.
<u>7198899</u>	April 2007	Schleyer et al.
<u>7204431</u>	April 2007	Li et al.
<u>7229770</u>	June 2007	Price et al.
<u>7252943</u>	August 2007	Griffiths et al.
<u>7267938</u>	September 2007	Anderson et al.

<u>7268167</u>	September 2007	Higuchi et al.
<u>7282337</u>	October 2007	Harris
<u>7291462</u>	November 2007	O'Brien et al.
<u>7294503</u>	November 2007	Quake et al.
<u>7300765</u>	November 2007	Patel
<u>7308364</u>	December 2007	Shaughnessy et al.
<u>7314721</u>	January 2008	Gure et al.
<u>7316906</u>	January 2008	Chiorazzi et al.
<u>7326529</u>	February 2008	Ali et al.
<u>7332280</u>	February 2008	Levy et al.
<u>7332590</u>	February 2008	Nacht et al.
<u>7341211</u>	March 2008	Ganan Calvo et al.
<u>7348142</u>	March 2008	Wang
<u>7358231</u>	April 2008	McCaffey et al.
<u>7361474</u>	April 2008	Siegler
<u>7364862</u>	April 2008	Ali et al.
<u>7368255</u>	May 2008	Bae et al.
<u>7378233</u>	May 2008	Sidransky et al.
<u>7378280</u>	May 2008	Quake et al.
<u>7390463</u>	June 2008	He et al.
<u>7393665</u>	July 2008	Brenner
<u>7416851</u>	August 2008	Davi et al.
<u>7429467</u>	September 2008	Holliger et al.
<u>7432064</u>	October 2008	Salceda et al.
<u>7442507</u>	October 2008	Polsky et al.
<u>7449303</u>	November 2008	Coignet
<u>7468271</u>	December 2008	Golovchenko et al.
<u>7473530</u>	January 2009	Huttemann
<u>7473531</u>	January 2009	Domon et al.
<u>7476506</u>	January 2009	Schleyer et al.
<u>7479370</u>	January 2009	Coignet
<u>7479371</u>	January 2009	Ando et al.
<u>7479376</u>	January 2009	Waldman et al.
<u>7482129</u>	January 2009	Soyupak et al.
<u>7501244</u>	March 2009	Reinhard et al.
<u>7504214</u>	March 2009	Erlander et al.
<u>7507532</u>	March 2009	Chang et al.
<u>7507541</u>	March 2009	Raitano et al.

<u>7510707</u>	March 2009	Platica et al.
<u>7510842</u>	March 2009	Podust et al.
<u>7514209</u>	April 2009	Dai et al.
<u>7514210</u>	April 2009	Holliger et al.
<u>7524633</u>	April 2009	Sidransky
<u>7527933</u>	May 2009	Sahin et al.
<u>7537897</u>	May 2009	Brenner et al.
<u>7541383</u>	June 2009	Fu et al.
<u>7544473</u>	June 2009	Brenner
<u>7556776</u>	July 2009	Fraden et al.
<u>7582446</u>	September 2009	Griffiths et al.
<u>7622081</u>	November 2009	Chou et al.
<u>7632562</u>	December 2009	Nair et al.
<u>7635562</u>	December 2009	Harris et al.
<u>7638276</u>	December 2009	Griffiths et al.
<u>7655435</u>	February 2010	Holliger et al.
<u>7655470</u>	February 2010	Ismagilov et al.
<u>7666593</u>	February 2010	Lapidus
<u>7691576</u>	April 2010	Holliger et al.
<u>7698287</u>	April 2010	Becker et al.
<u>7708949</u>	May 2010	Stone et al.
<u>7718578</u>	May 2010	Griffiths et al.
<u>7736890</u>	June 2010	Sia et al.
<u>7741130</u>	June 2010	Lee, Jr. et al.
<u>7814175</u>	October 2010	Chang et al.
<u>7824889</u>	November 2010	Vogelstein et al.
<u>7888017</u>	February 2011	Quake et al.
<u>7897044</u>	March 2011	Hoyos et al.
<u>7897341</u>	March 2011	Griffiths et al.
<u>7901939</u>	March 2011	Ismagilov et al.
<u>7968287</u>	June 2011	Griffiths et al.
<u>8012382</u>	September 2011	Kim et al.
<u>8153402</u>	April 2012	Holliger et al.
<u>2001/0010338</u>	August 2001	Ganan-Calvo
<u>2001/0020011</u>	September 2001	Mathiowitz et al.
<u>2001/0023078</u>	September 2001	Bawendi et al.
<u>2001/0029983</u>	October 2001	Unger et al.
<u>2001/0034031</u>	October 2001	Short et al.

<u>2001/0041343</u>	November 2001	Pankowsky
<u>2001/0041344</u>	November 2001	Sepetov et al.
<u>2001/0042793</u>	November 2001	Ganan-Calvo
<u>2001/0048900</u>	December 2001	Bardell et al.
<u>2001/0050881</u>	December 2001	Depaoli et al.
<u>2002/0004532</u>	January 2002	Matathia et al.
<u>2002/0005354</u>	January 2002	Spence et al.
<u>2002/0008028</u>	January 2002	Jacobson et al.
<u>2002/0012971</u>	January 2002	Mehta
<u>2002/0022038</u>	February 2002	Biatry et al.
<u>2002/0022261</u>	February 2002	Anderson et al.
<u>2002/0033422</u>	March 2002	Ganan-Calvo
<u>2002/0036139</u>	March 2002	Becker et al.
<u>2002/0058332</u>	May 2002	Quake et al.
<u>2002/0067800</u>	June 2002	Newman et al.
<u>2002/0119459</u>	August 2002	Griffiths
<u>2002/0143437</u>	October 2002	Handique et al.
<u>2002/0155080</u>	October 2002	Glenn et al.
<u>2002/0158027</u>	October 2002	Moon et al.
<u>2002/0164271</u>	November 2002	Ho
<u>2002/0164629</u>	November 2002	Quake et al.
<u>2003/0012586</u>	January 2003	Iwata et al.
<u>2003/0015425</u>	January 2003	Bohm et al.
<u>2003/0017579</u>	January 2003	Corn et al.
<u>2003/0039169</u>	February 2003	Ehrfeld et al.
<u>2003/0059764</u>	March 2003	Ravkin et al.
<u>2003/0061687</u>	April 2003	Hansen et al.
<u>2003/0064414</u>	April 2003	Benecky et al.
<u>2003/0082795</u>	May 2003	Shuler et al.
<u>2003/0124586</u>	July 2003	Griffiths et al.
<u>2003/0144260</u>	July 2003	Gilon
<u>2003/0148544</u>	August 2003	Nie et al.
<u>2003/0183525</u>	October 2003	Elrod et al.
<u>2003/0224509</u>	December 2003	Moon et al.
<u>2003/0229376</u>	December 2003	Sandhu
<u>2003/0230486</u>	December 2003	Chien et al.
<u>2003/0232356</u>	December 2003	Dooley et al.

<u>2004/0005582</u>	January 2004	Shipwash
<u>2004/0005594</u>	January 2004	Holliger et al.
<u>2004/0018525</u>	January 2004	Wirtz et al.
<u>2004/0027915</u>	February 2004	Lowe et al.
<u>2004/0037813</u>	February 2004	Simpson et al.
<u>2004/0041093</u>	March 2004	Schultz et al.
<u>2004/0050946</u>	March 2004	Wang et al.
<u>2004/0053247</u>	March 2004	Cordon-Cardo et al.
<u>2004/0068019</u>	April 2004	Higuchi et al.
<u>2004/0071781</u>	April 2004	Chattopadhyay et al.
<u>2004/0079881</u>	April 2004	Fischer et al.
<u>2004/0096515</u>	May 2004	Bausch et al.
<u>2004/0136497</u>	July 2004	Meldrum et al.
<u>2004/0146921</u>	July 2004	Eveleigh et al.
<u>2004/0159633</u>	August 2004	Whitesides et al.
<u>2004/0181131</u>	September 2004	Maynard et al.
<u>2004/0181343</u>	September 2004	Wigstrom et al.
<u>2004/0182712</u>	September 2004	Basol
<u>2004/0188254</u>	September 2004	Spaid
<u>2004/0224419</u>	November 2004	Zheng et al.
<u>2004/0253731</u>	December 2004	Holliger et al.
<u>2004/0258203</u>	December 2004	Yamano et al.
<u>2005/0032238</u>	February 2005	Karp et al.
<u>2005/0032240</u>	February 2005	Lee et al.
<u>2005/0037392</u>	February 2005	Griffiths et al.
<u>2005/0042648</u>	February 2005	Griffiths et al.
<u>2005/0048467</u>	March 2005	Sastry et al.
<u>2005/0064460</u>	March 2005	Holliger et al.
<u>2005/0069920</u>	March 2005	Griffiths et al.
<u>2005/0079510</u>	April 2005	Berka et al.
<u>2005/0084923</u>	April 2005	Mueller et al.
<u>2005/0087122</u>	April 2005	Ismaglov et al.
<u>2005/0095611</u>	May 2005	Chan et al.
<u>2005/0100895</u>	May 2005	Waldman et al.
<u>2005/0129582</u>	June 2005	Breidford et al.
<u>2005/0152908</u>	July 2005	Liew et al.
<u>2005/0164239</u>	July 2005	Griffiths et al.

<u>2005/0170431</u>	August 2005	Ibrahim et al.
<u>2005/0172476</u>	August 2005	Stone et al.
<u>2005/0183995</u>	August 2005	Deshpande et al.
<u>2005/0207940</u>	September 2005	Butler et al.
<u>2005/0221339</u>	October 2005	Griffiths et al.
<u>2005/0226742</u>	October 2005	Unger et al.
<u>2005/0227264</u>	October 2005	Nobile et al.
<u>2005/0260566</u>	November 2005	Fischer et al.
<u>2005/0272159</u>	December 2005	Ismagilov et al.
<u>2006/0003347</u>	January 2006	Griffiths et al.
<u>2006/0003429</u>	January 2006	Frost et al.
<u>2006/0003439</u>	January 2006	Ismagilov et al.
<u>2006/0036348</u>	February 2006	Handique et al.
<u>2006/0046257</u>	March 2006	Pollock et al.
<u>2006/0051329</u>	March 2006	Lee et al.
<u>2006/0078888</u>	April 2006	Griffiths et al.
<u>2006/0078893</u>	April 2006	Griffiths et al.
<u>2006/0094119</u>	May 2006	Ismagilov et al.
<u>2006/0108012</u>	May 2006	Barrow et al.
<u>2006/0110759</u>	May 2006	Paris et al.
<u>2006/0115821</u>	June 2006	Einstein et al.
<u>2006/0147909</u>	July 2006	Rarbach et al.
<u>2006/0153924</u>	July 2006	Griffiths et al.
<u>2006/0154298</u>	July 2006	Griffiths et al.
<u>2006/0160762</u>	July 2006	Zetter et al.
<u>2006/0163385</u>	July 2006	Link et al.
<u>2006/0169800</u>	August 2006	Rosell et al.
<u>2006/0195269</u>	August 2006	Yeatman et al.
<u>2006/0223127</u>	October 2006	Yip et al.
<u>2006/0234254</u>	October 2006	An et al.
<u>2006/0234259</u>	October 2006	Rubin et al.
<u>2006/0252057</u>	November 2006	Raponi et al.
<u>2006/0258841</u>	November 2006	Michl et al.
<u>2006/0263888</u>	November 2006	Fritz et al.
<u>2006/0269558</u>	November 2006	Murphy et al.
<u>2006/0269971</u>	November 2006	Diamandis
<u>2006/0281089</u>	December 2006	Gibson et al.
<u>2007/0003442</u>	January 2007	Link et al.

<u>2007/0026439</u>	February 2007	Faulstich et al.
<u>2007/0053896</u>	March 2007	Ahmed et al.
<u>2007/0054119</u>	March 2007	Garstecki et al.
<u>2007/0056853</u>	March 2007	Aizenberg et al.
<u>2007/0077572</u>	April 2007	Tawfik et al.
<u>2007/0077579</u>	April 2007	Griffiths et al.
<u>2007/0092914</u>	April 2007	Griffiths et al.
<u>2007/0120899</u>	May 2007	Ohnishi et al.
<u>2007/0154889</u>	July 2007	Wang
<u>2007/0166705</u>	July 2007	Milton et al.
<u>2007/0184439</u>	August 2007	Guilford et al.
<u>2007/0184489</u>	August 2007	Griffiths et al.
<u>2007/0195127</u>	August 2007	Ahn et al.
<u>2007/0259351</u>	November 2007	Chinitz et al.
<u>2007/0259368</u>	November 2007	An et al.
<u>2007/0259374</u>	November 2007	Griffiths et al.
<u>2007/0292869</u>	December 2007	Becker et al.
<u>2008/0003142</u>	January 2008	Link et al.
<u>2008/0009005</u>	January 2008	Kruk
<u>2008/0014589</u>	January 2008	Link et al.
<u>2008/0014590</u>	January 2008	Dahary et al.
<u>2008/0020940</u>	January 2008	Stedronsky et al.
<u>2008/0021330</u>	January 2008	Hwang et al.
<u>2008/0023330</u>	January 2008	Viovy et al.
<u>2008/0038754</u>	February 2008	Farias-Eisner et al.
<u>2008/0044828</u>	February 2008	Kwok
<u>2008/0050378</u>	February 2008	Nakamura et al.
<u>2008/0050723</u>	February 2008	Belacel et al.
<u>2008/0053205</u>	March 2008	Pollack et al.
<u>2008/0057514</u>	March 2008	Goldenring
<u>2008/0058432</u>	March 2008	Wang et al.
<u>2008/0063227</u>	March 2008	Rohrseitz
<u>2008/0064047</u>	March 2008	Zetter et al.
<u>2008/0081330</u>	April 2008	Kahvejian
<u>2008/0081333</u>	April 2008	Mori et al.
<u>2008/0092973</u>	April 2008	Lai
<u>2008/0113340</u>	May 2008	Schlegel
<u>2008/0118462</u>	May 2008	Alani et al.

<u>2008/0138806</u>	June 2008	Chow et al.
<u>2008/0166772</u>	July 2008	Hollinger et al.
<u>2008/0171078</u>	July 2008	Gray
<u>2008/0176211</u>	July 2008	Spence et al.
<u>2008/0176236</u>	July 2008	Tsao et al.
<u>2008/0181850</u>	July 2008	Thaxton et al.
<u>2008/0206756</u>	August 2008	Lee et al.
<u>2008/0222741</u>	September 2008	Chinnaiyan
<u>2008/0234138</u>	September 2008	Shaughnessy et al.
<u>2008/0234139</u>	September 2008	Shaughnessy et al.
<u>2008/0268473</u>	October 2008	Moses et al.
<u>2008/0269157</u>	October 2008	Srivastava et al.
<u>2008/0274908</u>	November 2008	Chang
<u>2008/0280302</u>	November 2008	Kebebew
<u>2008/0286199</u>	November 2008	Livingston et al.
<u>2008/0286801</u>	November 2008	Arjol et al.
<u>2008/0286811</u>	November 2008	Moses et al.
<u>2008/0293578</u>	November 2008	Shaughnessy et al.
<u>2008/0311570</u>	December 2008	Lai
<u>2008/0311604</u>	December 2008	Elting et al.
<u>2009/0004687</u>	January 2009	Mansfield et al.
<u>2009/0005254</u>	January 2009	Griffiths et al.
<u>2009/0012187</u>	January 2009	Chu et al.
<u>2009/0017463</u>	January 2009	Bhowmick
<u>2009/0021728</u>	January 2009	Heinz et al.
<u>2009/0023137</u>	January 2009	Van Der Zee et al.
<u>2009/0026082</u>	January 2009	Rothberg et al.
<u>2009/0029372</u>	January 2009	Wewer
<u>2009/0042737</u>	February 2009	Katz et al.
<u>2009/0053700</u>	February 2009	Griffiths et al.
<u>2009/0053732</u>	February 2009	Vermesh et al.
<u>2009/0060797</u>	March 2009	Mathies et al.
<u>2009/0062144</u>	March 2009	Guo
<u>2009/0068170</u>	March 2009	Weitz et al.
<u>2009/0075265</u>	March 2009	Budiman et al.
<u>2009/0075307</u>	March 2009	Fischer et al.
<u>2009/0075311</u>	March 2009	Karl

<u>2009/0081237</u>	March 2009	D'Andrea et al.
<u>2009/0081685</u>	March 2009	Beyer et al.
<u>2009/0087849</u>	April 2009	Malinowski et al.
<u>2009/0092973</u>	April 2009	Erlander et al.
<u>2009/0098542</u>	April 2009	Budiman et al.
<u>2009/0098543</u>	April 2009	Budiman et al.
<u>2009/0118128</u>	May 2009	Liu et al.
<u>2009/0124569</u>	May 2009	Bergan et al.
<u>2009/0127454</u>	May 2009	Ritchie et al.
<u>2009/0127589</u>	May 2009	Rothberg et al.
<u>2009/0131353</u>	May 2009	Insel et al.
<u>2009/0131543</u>	May 2009	Weitz et al.
<u>2009/0191565</u>	July 2009	Lapidus et al.
<u>2009/0197248</u>	August 2009	Griffiths et al.
<u>2009/0197772</u>	August 2009	Griffiths et al.
<u>2009/0246788</u>	October 2009	Albert et al.
<u>2009/0325236</u>	December 2009	Griffiths et al.
<u>2010/0003687</u>	January 2010	Simen et al.
<u>2010/0009353</u>	January 2010	Barnes et al.
<u>2010/0022414</u>	January 2010	Link et al.
<u>2010/0035252</u>	February 2010	Rothberg et al.
<u>2010/0075436</u>	March 2010	Urdea et al.
<u>2010/0105112</u>	April 2010	Holtze et al.
<u>2010/0111768</u>	May 2010	Banerjee et al.
<u>2010/0124759</u>	May 2010	Wang et al.
<u>2010/0136544</u>	June 2010	Agresti et al.
<u>2010/0137143</u>	June 2010	Rothberg et al.
<u>2010/0137163</u>	June 2010	Link et al.
<u>2010/0159592</u>	June 2010	Holliger et al.
<u>2010/0172803</u>	July 2010	Stone et al.
<u>2010/0188073</u>	July 2010	Rothberg et al.
<u>2010/0197507</u>	August 2010	Rothberg et al.
<u>2010/0210479</u>	August 2010	Griffiths et al.
<u>2010/0213628</u>	August 2010	Bausch et al.
<u>2010/0233026</u>	September 2010	Ismagliov et al.
<u>2010/0282617</u>	November 2010	Rothberg et al.
<u>2010/0300559</u>	December 2010	Schultz et al.

<u>2010/0300895</u>	December 2010	Nobile et al.
<u>2010/0301398</u>	December 2010	Rothberg et al.
<u>2010/0304982</u>	December 2010	Hinz et al.
<u>2011/0000560</u>	January 2011	Miller et al.
<u>2011/0142734</u>	June 2011	Ismagilov et al.
<u>2011/0174622</u>	July 2011	Ismagilov et al.
<u>2011/0176966</u>	July 2011	Ismagilov et al.
<u>2011/0177494</u>	July 2011	Ismagilov et al.
<u>2011/0177586</u>	July 2011	Ismagilov et al.
<u>2011/0177609</u>	July 2011	Ismagilov et al.
<u>2011/0188717</u>	August 2011	Baudry et al.
<u>2011/0190146</u>	August 2011	Boehm et al.
<u>2011/0244455</u>	October 2011	Larson et al.
<u>2011/0250597</u>	October 2011	Larson et al.
<u>2011/0275063</u>	November 2011	Weitz et al.
<u>2012/0010098</u>	January 2012	Griffiths et al.
<u>2012/0015382</u>	January 2012	Weitz et al.
<u>2012/0015822</u>	January 2012	Weitz et al.

Foreign Patent Documents

2004225691	Jun 2010	AU
2520548	Oct 2004	CA
563807	Jul 1975	CH
4308839	Apr 1997	DE
0047130	Feb 1985	EP
0249007	Mar 1991	EP
0476178	Mar 1992	EP
0540281	Jul 1996	EP
0528580	Dec 1996	EP
0895120	Feb 1999	EP
1741482	Jan 2007	EP
2127736	Dec 2009	EP
0114854.3	Apr 1969	GB
1446998	Aug 1976	GB
2005224	Apr 1979	GB
2047880	Dec 1980	GB
2062225	May 1981	GB
2064114	Jun 1981	GB
2097692	Nov 1982	GB

0221053.2	Jun 1989	GB
3-232525	Oct 1998	JP
2000271475	Oct 2000	JP
WO-84/02000	May 1984	WO
WO-91/05058	Apr 1991	WO
WO-91/07772	May 1991	WO
WO-92/03734	Mar 1992	WO
WO-92/21746	Dec 1992	WO
WO-93/03151	Feb 1993	WO
WO-93/08278	Apr 1993	WO
WO-93/22053	Nov 1993	WO
WO-93/22054	Nov 1993	WO
WO-93/22055	Nov 1993	WO
WO-93/22058	Nov 1993	WO
WO-93/22421	Nov 1993	WO
WO-94/16332	Jul 1994	WO
WO-94/23738	Oct 1994	WO
WO-94/24314	Oct 1994	WO
WO-94/26766	Nov 1994	WO
WO-98/00705	Jan 1995	WO
WO-95/11922	May 1995	WO
WO-95/19922	Jul 1995	WO
WO-95/24929	Sep 1995	WO
WO-95/33447	Dec 1995	WO
WO-96/34112	Oct 1996	WO
WO-96/38730	Dec 1996	WO
WO-96/40062	Dec 1996	WO
WO-96/40723	Dec 1996	WO
WO-97/00125	Jan 1997	WO
WO-97/00442	Jan 1997	WO
WO-97/04297	Feb 1997	WO
WO-97/04748	Feb 1997	WO
WO-97/23140	Jul 1997	WO
WO-97/28556	Aug 1997	WO
WO-97/39814	Oct 1997	WO
WO-97/40141	Oct 1997	WO
WO-97/45644	Dec 1997	WO
WO-97/47763	Dec 1997	WO

WO-98/00231	Jan 1998	WO
WO-98/02237	Jan 1998	WO
WO-98/10267	Mar 1998	WO
WO-98/13502	Apr 1998	WO
WO-98/23733	Jun 1998	WO
WO-98/31700	Jul 1998	WO
WO-98/33001	Jul 1998	WO
WO-98/34120	Aug 1998	WO
WO-98/37186	Aug 1998	WO
WO-98/41869	Sep 1998	WO
WO-98/52691	Nov 1998	WO
WO-98/58085	Dec 1998	WO
WO-99/02671	Jan 1999	WO
WO-99/22858	May 1999	WO
WO-99/28020	Jun 1999	WO
WO-99/31019	Jun 1999	WO
WO-99/54730	Oct 1999	WO
WO-99/61886	Dec 1999	WO
WO-00/04139	Jan 2000	WO
WO-00/47322	Feb 2000	WO
WO-00/52455	Feb 2000	WO
WO-00/40712	Jun 2000	WO
WO-00/61275	Oct 2000	WO
WO-00/70080	Nov 2000	WO
WO-00/76673	Dec 2000	WO
WO-01/12327	Feb 2001	WO
WO-01/14589	Mar 2001	WO
WO-01/16244	Mar 2001	WO
WO-01/64332	Sep 2001	WO
WO-01/68257	Sep 2001	WO
WO-01/69289	Sep 2001	WO
WO-01/72431	Oct 2001	WO
WO-01/80283	Oct 2001	WO
WO-02/18949	Mar 2002	WO
WO-02/22869	Mar 2002	WO
WO-02/23163	Mar 2002	WO
WO-02/31203	Apr 2002	WO
WO-02/47665	Jun 2002	WO

WO-02/47665	Aug 2002	WO
WO-02/060275	Aug 2002	WO
WO-02/078845	Oct 2002	WO
WO-02/103011	Dec 2002	WO
WO-02/103363	Dec 2002	WO
WO-03/011443	Feb 2003	WO
WO-03/037302	May 2003	WO
WO-03/044187	May 2003	WO
WO-03/078659	Sep 2003	WO
WO-03/099843	Dec 2003	WO
WO-2004/002627	Jan 2004	WO
WO-2004/018497	Mar 2004	WO
WO-2004/024917	Mar 2004	WO
WO-2004/038363	May 2004	WO
WO-2004/069849	Aug 2004	WO
WO-2004/074504	Sep 2004	WO
WO-2004/083443	Sep 2004	WO
WO-2004/087308	Oct 2004	WO
WO-2004/088314	Oct 2004	WO
WO-2004/091763	Oct 2004	WO
WO-2004/102204	Nov 2004	WO
WO-2004/103565	Dec 2004	WO
WO-2005/000970	Jan 2005	WO
WO-2005/002730	Jan 2005	WO
WO-2005/021151	Mar 2005	WO
WO-2005/103106	Nov 2005	WO
WO-2005/118138	Dec 2005	WO
WO-2006/002641	Jan 2006	WO
WO-2006/009657	Jan 2006	WO
WO-2006/027757	Mar 2006	WO
WO-2006/038035	Apr 2006	WO
WO-2006/040551	Apr 2006	WO
WO-2006/040554	Apr 2006	WO
WO-2006/078841	Jul 2006	WO
WO-2006/096571	Sep 2006	WO
WO-2006/101851	Sep 2006	WO
WO-2007/021343	Feb 2007	WO
WO-2007/030501	Mar 2007	WO

WO-2007/081385	Jul 2007	WO
WO-2007/081387	Jul 2007	WO
WO-2007/089541	Aug 2007	WO
WO-2007/114794	Oct 2007	WO
WO-2007/123744	Nov 2007	WO
WO-2007/133710	Nov 2007	WO
WO-2007/138178	Dec 2007	WO
WO-2008/021123	Feb 2008	WO
WO-2008/063227	May 2008	WO
WO-2008/097559	Aug 2008	WO
WO-2008/121342	Oct 2008	WO
WO-2008/130623	Oct 2008	WO
WO-2009/029229	Mar 2009	WO
WO-2010/056728	May 2010	WO
WO-2010/040006	Aug 2010	WO
WO-2010/151776	Dec 2010	WO
WO-2011/042564	Apr 2011	WO
WO-2011/079176	Jun 2011	WO

Other References

Kiss et al. Anal. Chem. 2008, vol. 80, p. 8975-8981. cited by examiner .

Tewhey et al. Nature Biotechnology, 2009, vol. 27(11). p. 1025-1031. cited by examiner .

Adang, A.E. et al., The contribution of combinatorial chemistry to lead generation: an interim analysis, Curr Med Chem 8: 985-998 (2001). cited by applicant .

Advisory Action for U.S. Appl. No. 11/360,845, mailed Jun. 14, 2010. cited by applicant .

Advisory Action for U.S. Appl. No. 11/698,298 mailed May 20, 2011. cited by applicant .

Affholter and F. Arnold, Engineering a Revolution, Chemistry in Britain, Apr. 1999, p. 48. cited by applicant .

Agrawal and Tang, Site-specific functionalization of oligodeoxynucleotides for non-radioactive labelling, Tetrahedron Letters 31:1543-1546 (1990). cited by applicant .

Aharoni et al., High-Throughput screens and selections of enzyme-encoding genes, Curr Opin Chem Biol, 9(2): 210-6 (2005). cited by applicant .

Ahn et al., Dielectrophoretic manipulation of drops for high-speed microfluidic sorting devices, Applied Phys Lett 88, 024104 (2006). cited by applicant .

Allen et al., High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors J Biomol Screen. 5(2):63-9 (2000). cited by applicant .

Altman et al., Solid-state laser using a rhodamine-doped silica gel compound, IEEE Photonics technology letters 3(3):189-190 (1991). cited by applicant .

Amstutz, P. et al., In vitro display technologies: novel developments and applications. Curr Opin Biotechnol, 12, 400-405 (2001). cited by applicant .

- Anarbaev et al., Klenow fragment and DNA polymerase alpha-primase from *Servus* calf thymus in water-in-oil microemulsions, *Biochim Biophys Acta* 1384:315-324 (1998). cited by applicant .
- Anderson et al., Preparation of a cell-free protein-synthesizing system from wheat germ, *Methods Enzymol* 101:635-44 (1983). cited by applicant .
- Anderson, J.E., Restriction endonucleases and modification methylases, *Curr. Op. Struct. Biol.*, 3:24-30 (1993). cited by applicant .
- Ando, S. et al., PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization, *J Pharm Sci*, 88(1):126-130 (1999). cited by applicant .
- Angell et al., Silicon micromechanical devices, *Scientific American* 248:44-55 (1983). cited by applicant .
- Anhuf et al., Determination of SMN1 and SMN2 copy number using TaqMan technology, *Hum Mutat* 22(1):74-78 (2003). cited by applicant .
- Anna et al., Formation of dispersions using flow focusing in microchannels, *Applied Physics Letters*, 82(3): 364-366 (2003). cited by applicant .
- Arkin, M.R. et al., Probing the importance of second sphere residues in an esterolytic antibody by phage display, *J Mol Biol* 284(4):1083-94 (1998). cited by applicant .
- Armstrong et al., Multiple-Component Condensation Strategies for Combinatorial Library Synthesis, *Acc. Chem. Res.* 29(3):123-131 (1996). cited by applicant .
- Ashkin and Dziedzic, Optical trapping and manipulation of viruses and bacteria, *Science* 235(4795):1517-20 (1987). cited by applicant .
- Ashkin et al., Optical trapping and manipulation of single cells using infrared laser beams, *Nature* 330:769-771 (1987). cited by applicant .
- Atwell, S. & Wells, J.A., Selection for Improved Subtiligases by Phage Display, *PNAS* 96: 9497-9502(1999). cited by applicant .
- Auroux, Pierre-Alain et al., Micro Total Analysis Systems. 2. Analytical Standard Operations and Applications, *Analytical Chemistry*, vol. 74, No. 12, 2002, pp. 2637-2652. cited by applicant .
- Baccarani et al., *Escherichia coli* dihydrofolate reductase: isolation and characterization of two isozymes, *Biochemistry* 16(16):3566-72 (1977). cited by applicant .
- Baez et al., Glutathione transferases catalyze the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes, *Biochem. J* 324:25-28 (1997). cited by applicant .
- Bagwe et al., Improved drug delivery using microemulsions: rationale, recent progress, and new horizons, *Crit Rev Ther Drug Carr Sys* 18(1):77-140 (2001). cited by applicant .
- Baker, M., Clever PCR: more genotyping, smaller volumes, *Nature Methods* 7:351-356 (2010). cited by applicant .
- Ball and Schwartz, CMATRIX: software for physiologically based pharmacokinetic modeling using a symbolic matrix representation system, *Comput Biol Med* 24(4):269-76 (1994). cited by applicant .
- Ballantyne and Nixon, Selective Area Metallization by Electron-Beam Controlled Direct Metallic Deposition, *J. Vac. Sci. Technol.* 10:1094 (1973). cited by applicant .
- Barany F., The ligase chain reaction in a PCR World, *PCR Methods and Applications* 1(1):5-16 (1991). cited by applicant .
- Barany, F. Genetic disease detection and DNA amplification using cloned thermostable ligase, *PNAS* 88(1): 189-93 (1991). cited by applicant .

- Baret et al., Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity, *Lab on a Chip* 9:1850-1858 (2009). cited by applicant .
- Baret et al., Kinetic aspects of emulsion stabilization by surfactants: a microfluidic analysis, *Langmuir* 25:6088-6093 (2009). cited by applicant .
- Bass et al., Hormone Phage: An Enrichment Method for Variant Proteins With Altered Binding Properties, *Proteins* 8:309-314(1990). cited by applicant .
- Bauer, J., Advances in cell separation: recent developments in counterflow centrifugal elutriation and continuous flow cell separation, *J Chromatography*, 722:55-69 (1999). cited by applicant .
- Beebe et al., Functional hydrogel structures for autonomous flow control inside microfluidic channels, *Nature* 404:588-590 (2000). cited by applicant .
- Beer et al., On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets, *Anal. Chem.*, 2007, v. 79, pp. 847-8475. cited by applicant .
- Bein, Thomas, Efficient Assays for Combinatorial methods for the Discovery of Catalysts, *Agnew. Chem. Int. Ed.* 38:3, 323-26 (1999). cited by applicant .
- Benichou et al., Double Emulsions Stabilized by New Molecular Recognition Hybrids of Natural Polymers, *Polym. Adv. Tehcnol* 13:1019-1031 (2002). cited by applicant .
- Benner, S.A., Expanding the genetic lexicon: incorporating non-standard amino acids into proteins by ribosome-based synthesis, *Trends Biotechnol* 12:158-63 (1994). cited by applicant .
- Benning, M.M. et al., The binding of substrate analogs to phosphotriesterase. *J Biol Chem*, 275, 30556-30560 (2000). cited by applicant .
- Berman et al., An agarose gel electrophoresis assay for the detection of DNA-binding activities in yeast cell extracts, *Methods Enzymol* 155:528-37 (1987). cited by applicant .
- Bernath et al, In Vitro Compartmentalization by Double Emulsions: Sorting and Gene Enrichment by Fluorescence Activated Cell Sorting, *Anal. Biochem* 325:151-157 (2004). cited by applicant .
- Bernath et al., Directed evolution of protein inhibitors of DNA-nucleases by in vitro compartmentalization (IVC) and nano-droplet delivery, *J. Mol. Biol* 345(5):1015-26 (2005). cited by applicant .
- Betlach, L. et al., A restriction endonuclease analysis of the bacterial plasmid controlling the EcoRI restriction and modification of DNA. *Federation Proceedings*, 35, 2037-2043 (1976). cited by applicant .
- Bibette et al., Emulsions: basic principles, *Rep. Prog. Phys.* 62: 969-1033 (1999). cited by applicant .
- Bico, Jose et al., Rise of Liquids and Bubbles in Angular Capillary Tubes, *Journal of Colloid and Interface Science*, 247:162-166 (2002). cited by applicant .
- Bico, Jose et al., Self-Propelling Slugs, *J. Fluid Mech.*, 467:101-127 (2002). cited by applicant .
- Blattner and Dahlberg, RNA synthesis startpoints in bacteriophage lambda: are the promoter and operator transcribed, *Nature New Biol* 237(77):227-32 (1972). cited by applicant .
- Boder et al., Yeast surface display for screening combinatorial polypeptide libraries, *Nat Biotechnol* 15(6):553-7 (1997). cited by applicant .
- Bougueleret, L. et al., Characterization of the gene coding for the EcoRV restriction and modification system of *Escherichia coli*, *Nucleic Acids Res*, 12(8):3659-76 (1984). cited by applicant .
- Boyum, A., Separation of leukocytes from blood and bone marrow. Introduction, *Scand J Clin Lab Invest Suppl* 97:7 (1968). cited by applicant .
- Branebjerg et al., Fast mixing by lamination, *MEMS Proceedings 9th Ann WO rkshop*, San Diego,

- Feb. 11-15, 1996, 9:441-446 (1996). cited by applicant .
- Braslavsky et al., Sequence information can be obtained from single DNA molecules, PNAS 100(7):3960-3964 (2003). cited by applicant .
- Bringer et al., Microfluidic Systems for Chemical Kinetics That Rely on Chaotic Mixing in Droplets, Philos Transact A Math Phys Eng Sci 362:1-18 (2004). cited by applicant .
- Brody et al., A self-assembled microlensing rotational probe, Applied Physics Letters, 74:144-46 (1999). cited by applicant .
- Brown et al., Chemical synthesis and cloning of a tyrosine tRNA gene, Methods Enzymol 68:109-151 (1979). cited by applicant .
- Bru, R. et al., Catalytic activity of elastase in reverse micelles, Biochem Mol Bio Int, 31(4):685-92 (1993). cited by applicant .
- Bru, R. et al., Product inhibition of alpha-chymotrypsin in reverse micelles. Eur J Biochem 199(1): 95-103 (1991). cited by applicant .
- Brummelkamp et al., A system for stable expression of short interfering RNAs in mammalian cells, Science 296(5567):550-3 (2002). cited by applicant .
- Buckpitt et al., Hepatic and pulmonary microsomal metabolism of naphthalene to glutathione adducts: factors affecting the relative rates of conjugate formation, J. Pharmacol. Exp. Ther. 231:291-300 (1984). cited by applicant .
- Buican et al., Automated single-cell manipulation and sorting by light trapping, Applied Optics 26(24):5311-5316 (1987). cited by applicant .
- Burbaum, J., Miniaturization technologies in HTS: how fast, how small, how soon Drug Discov Today 3:313-322 (1998). cited by applicant .
- Burns et al., Microfabricated structures for integrated DNA analysis, Proc. Natl. Acad. Sci. USA, 93:5556-5561(1996). cited by applicant .
- Burns, J.R. et al., The Intensification of Rapid Reactions in Multiphase Systems Using Slug Flow in Capillaries, Lab on a Chip, 1:10-15 (2001). cited by applicant .
- Burns, Mark et al., An Integrated Nanoliter DNA Analysis Device, Science, 282:484-487(1998). cited by applicant .
- Byrnes, P.J. et al., Sensitive fluorogenic substrates for the detection of trypsin-like proteases and pancreatic elastase, Anal Biochem, 126:447 (1982). cited by applicant .
- Cahill et al., Polymerase chain reaction and Q beta replicase amplification, Clin Chem 37(9):1482-5 (1991). cited by applicant .
- Caldwell, S.R. et al., Limits of diffusion in the hydrolysis of substrates by the phosphodiesterase from *Pseudomonas diminuta*, Biochemistry, 30: 7438-7444 (1991). cited by applicant .
- Calvert, P., Inkjet printing for materials and devices, Chem Mater 13: 3299-3305 (2001). cited by applicant .
- Caruthers, Gene synthesis machines: DNA chemistry and its uses, Science 230:281-285 (1985). cited by applicant .
- Chakrabarti, A.C. et al., Production of RNA by a polymerase protein encapsulated within phospholipid vesicles, J Mol Evol, 39(6):555-9 (1994). cited by applicant .
- Chamberlain and Ring, Characterization of T7-specific ribonucleic acid polymerase. 1. General properties of the enzymatic reaction and the template specificity of the enzyme, J Biol Chem 248:2235-44 (1973). cited by applicant .
- Chan, Emory M. et al., Size-Controlled Growth of CdSe Nanocrystals in Microfluidic Reactors, Nano Letters, 3(2):199-201(2003). cited by applicant .

- Chang and Su, Controlled double emulsification utilizing 3D PDMS microchannels, *Journal of Micromechanics and Microengineering* 18:1-8 (2008). cited by applicant .
- Chang, T.M., Recycling of NAD(P) by multienzyme systems immobilized by microencapsulation in artificial cells, *Methods Enzymol*, 136(67):67-82 (1987). cited by applicant .
- Chao et al., Control of Concentration and Volume Gradients in Microfluidic Droplet Arrays for Protein Crystallization Screening, 26th Annual International Conference of the IEEE Engineering in Medicine and Biology Society, San Francisco, California Sep. 1-5, 2004. cited by applicant .
- Chao et al., Droplet Arrays in Microfluidic Channels for Combinatorial Screening Assays, Hilton Head 2004: A Solid State Sensor, Actuator and Microsystems Workshop, Hilton Head Island, South Carolina, Jun. 6-10, (2004). cited by applicant .
- Chapman et al., In vitro selection of catalytic RNAs, *Curr. op. Struct. Biol*, 4:618-22 (1994). cited by applicant .
- Chayen, Crystallization with oils: a new dimension in macromolecular crystal growth *Journal of Crystal Growth*, 196:434-441 (1999). cited by applicant .
- Chen et al., Capturing a Photoexcited Molecular Structure Through Time-Domain X-ray Absorption Fine Structure, *Science* 292(5515):262-264 (2001). cited by applicant .
- Chen et al., Microfluidic Switch for Embryo and Cell Sorting The 12th International Conference on Solid State Sensors, Actuators, and Microsystems, Boston, MA Jun. 8-12, 2003 *Transducers*, 1: 659-662 (2003). cited by applicant .
- Chen-Goodspeed et al., Structural Determinants of the substrate and stereochemical specificity of phosphotriesterase, *Biochemistry*, 40(5):1325-31 (2001). cited by applicant .
- Chen-Goodspeed, M. et al., Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues, *Biochemistry*, 40: 1332-1339 (2001b). cited by applicant .
- Cheng, Z., et al, Electro flow focusing in microfluidic devices, *Microfluidics Poster*, presented at DBAS, *Frontiers in Nanoscience*, presented Apr. 10, 2003. cited by applicant .
- Chetverin and Spirin, Replicable RBA vectors: prospects for cell-free gene amplification, expression, and cloning, *Prog Nucleic Acid Res Mol Biol*, 51:225-70 (1995). cited by applicant .
- Chiang, C.M. et al., Expression and purification of general transcription factors by FLAG epitope-tagging and peptide elution, *Pept Res*, 6: 62-64 (1993). cited by applicant .
- Chiba et al., Controlled protein delivery from biodegradable tyrosine-containing poly(anhydride-co-imide) microspheres, *Biomaterials*, 18(13): 893-901 (1997). cited by applicant .
- Chiou et al., A closed-cycle capillary polymerase chain reaction machine, *Analytical Chemistry*, American Chemical Society, 73:2018-21 (2001). cited by applicant .
- Chiu et al., Chemical transformations in individual ultrasmall biomimetic containers, *Science*, 283: 1892-1895 (1999). cited by applicant .
- Chou et al., A microfabricated device for sizing and sorting DNA molecules 96:11-13 (1998). cited by applicant .
- Clackson, T. et al., In vitro selection from protein and peptide libraries, *Trends Biotechnol*, 12:173-84 (1994). cited by applicant .
- Clausell-Tormos et al., Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms, *Chem Biol* 15(5):427-437 (2008). cited by applicant .
- Cohen, S. et al., Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres, *Pharm Res*, 8(6):713-720 (1991). cited by applicant .
- Collins et al., Optimization of Shear Driven Droplet Generation in a Microfluidic Device, *ASME*

Demartis et al., A strategy for the isolation of catalytic activities from repertoires of enzymes

displayed on phage, *J. Mol. Biol* 286:617-633 (1999). cited by applicant .

Dickinson, E., Emulsions and droplet size control, Wedlock, D.J., Ed., in *Controlled Particle Droplet and Bubble Formulation*, Butterworth-Heinemann, 191-257 (1994). cited by applicant .

DiMatteo, et al., Genetic conversion of an SMN2 gene to SMN1: A novel approach to the treatment of spinal muscular atrophy, *Exp Cell Res.* 314(4):878-886 (2008). cited by applicant .

Dinsmore et al., Colioidosomes: Selectively Permeable Capsules Composed of Colloidal Particles, *Science* 298(5595):1006-1009. (2002). cited by applicant .

Dittrich et al., A new embedded process for compartmentalized cell-free protein expression and on-line detection in microfluidic devices, *ChemBiochem* 6(5):811-814 (2005). cited by applicant .

Doi et al., In vitro selection of restriction endonucleases by in vitro compartmentalization, *Nucleic Acids Res.* 32(12): e95 (2004). cited by applicant .

Doi, N. and Yanagawa, H. STABLE: protein-DNA fusion system for screening of combinatorial protein libraries in vitro, *FEBS Lett.*, 457: 227-230 (1999). cited by applicant .

Doman, T.N. et al., Molecular docking and high-throughput screening for novel inhibitors of protein tyrosine phosphatase-1B, *J Med Chem*, 45: 2213-2221 (2002). cited by applicant .

Domling A., Recent advances in isocyanide-based multicomponent chemistry, *Curr Opin Chem Biol*, 6(3):306-13 (2002). cited by applicant .

Domling and Ugi, Multicomponent Reactions with Isocyanides, *Angew Chem Int Ed* 39(18):3168-3210 (2000). cited by applicant .

Dove et al., In Brief, *Nature Biotechnology* 20:1213 (2002). cited by applicant .

Dower et al., High efficiency transformation of *E. coli* by high voltage electroporation, *Nucleic Acids Res* 16:6127-6145 (1988). cited by applicant .

Dressman et al., Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations, *PNAS* 100:8817-22 (2003). cited by applicant .

Dreyfus et al., Ordered and disordered patterns in two phase flows in microchannels, *Phys Rev Lett* 90(14):144505-1-144505-4 (2003). cited by applicant .

Drmanac et al., Sequencing by hybridization: towards an automated sequencing of one million M13 clones arrayed on membranes, *Electrophoresis* 13:566-573 (1992). cited by applicant .

Dubertret et al., In vivo imaging of quantum dots encapsulated in phospholipid micelles, *Science*, 298: 1759-1762 (2002). cited by applicant .

Duffy et al., Rapid Prototyping of Microfluidic Systems and Polydimethylsiloxane, *Anal Chem* 70:474-480 (1998). cited by applicant .

Duggleby, R. G. *Enzyme Kinetics and Mechanisms*, Pt D. Academic Press 249:61-90 (1995). cited by applicant .

Dumas, D.P., Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*, *J Biol Chem* 264: 19659-19665 (1989). cited by applicant .

Eckert and Kunkel, DNA polymerase fidelity and the polymerase chain reaction, *Genome Res* 1:17-24 (1991). cited by applicant .

Edd et al., Controlled encapsulation of single-cells into monodisperse picolitre drops, *Lab Chip* 8(8):1262-1264 (2008). cited by applicant .

Edel, Joshua B. et al., Microfluidic Routes to the Controlled Production of Nanoparticles, *Chemical Communications*, 1136-1137 (2002). cited by applicant .

Edris et al., Encapsulation of orange oil in a spray dried double emulsion, *Nahrung/Food*, 45(2):133-137 (2001). cited by applicant .

Effenhauser et al., Glass chips for high-speed capillary electrophoresis separations with

- submicrometer plate heights, *Anal Chem* 65:2637-2642 (1993). cited by applicant .
- Eggers, Jens et al., Coalescence of Liquid Drops, *J. Fluid Mech.*, 401 : 293-310 (1999). cited by applicant .
- Ehrig, T. et al., Green-fluorescent protein mutants with altered fluorescence excitation spectra, *Febs Lett*, 367(2):163-66 (1995). cited by applicant .
- Eigen et al., hypercycles and compartments: compartments assists--but does not replace-- hypercyclic organization of early genetic information, *J Theor Biol*, 85:407-11 (1980). cited by applicant .
- Eigen et al., The hypercycle: coupling of RNA and protein biosynthesis in the infection cycle of an RNA bacteriophage, *Biochemistry*, 30:11005-18 (1991). cited by applicant .
- Eigen, Wie entsteht information Prinzipien der selbstorganisation in der biologie, *Berichte der punsen-gesellschaft für physikalische chemi*, 80:1059-81 (1976). cited by applicant .
- Ellington and Szostak, In vitro selection of RNA molecules that bind specific ligands, *Nature*, 346:818-822 (1990). cited by applicant .
- Ellman et al., Biosynthetic method for introducing unnatural amino acids site-specifically into proteins, *Methods Enzymol*, 202:301-36 (1991). cited by applicant .
- Endo et al. Kinetic determination of trace cobalt by visual autocatalytic indication, *Talanta* 47:349-353 (1998). cited by applicant .
- Endo et al., Autocatalytic decomposition of cobalt complexes as an indicator system for the determination of trace amounts of cobalt and effectors, *Analyst* 121:391-394 (1996). cited by applicant .
- Eow et al., Electrocoalesce-separators for the separation of aqueous drops from a flowing dielectric viscous liquid, *Separation and Purification Tech* 29:63-77 (2002). cited by applicant .
- Eow et al., Electrostatic enhancement of coalescence of water droplets in oil: a review of the technology, *Chemical Engineering Journal* 85:357-368 (2002). cited by applicant .
- Eow et al., Motion, deformation and break-up of aqueous drops in oils under high electric field strengths, *Chemical Eng Proc* 42:259-272 (2003). cited by applicant .
- Eow et al., The behavior of a liquid-liquid interface and drop-interface coalescence under the influence of an electric field, *Colloids and Surfaces A: Physiochem. Eng. Aspects* 215:101-123 (2003). cited by applicant .
- Eow, et al. Electrostatic and hydrodynamic separation of aqueous drops in a flowing viscous oil, *Chemical Eng Proc* 41:649-657 (2002). cited by applicant .
- Extended European Search Report for EP 10181911.8 mailed Jun. 1, 2011 (7 pages). cited by applicant .
- Extended European Search Report for EP 10184514.7 mailed Dec. 20, 2010 (5 pages). cited by applicant .
- Faca et al., A mouse to human search for plasma proteome changes associated with pancreatic tumor development, *PLoS Med* 5(6):e123 (2008). cited by applicant .
- Fahy et al., Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR, *PCR Methods Appl* 1:25-33 (1991). cited by applicant .
- Fan and Harrison, Micromachining of capillary electrophoresis injectors and separators on glass chips and evaluation of flow at capillary intersections, *Anal Chem* 66:177-184 (1994). cited by applicant .
- Fastrez, J., In vivo versus in vitro screening or selection for catalytic activity in enzymes and abzymes, *Mol Biotechnol* 7(1):37-55 (1997). cited by applicant .

Fettinger et al., Stacked modules for micro flow systems in chemical analysis: concept and studies using an enlarged model, *Sens Actuat B*, 17:19-25 (1993). cited by applicant .

Fiedler et al., Dielectrophoretic sorting of particles and cells in a microsystem, *Anal Chem* 70(9):1909-1915 (1998). cited by applicant .

Field, J. et al., Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol Cell Biol*, 8: 2159-2165 (1988). cited by applicant .

Fields, S. and Song, O., A novel genetic system to detect protein-protein interactions, *Nature* 340(6230): 245-6 (1989). cited by applicant .

Filella et al., TAG-72, CA 19.9 and CEA as tumor markers in gastric cancer, *Acta Oncol* 33(7):747-751 (1994). cited by applicant .

Finch, C.A., Encapsulation and controlled release, *Spec Publ R Soc Chem*, 138:35 (1993). cited by applicant .

Finch, C.A., Industrial Microencapsulation: Polymers for Microcapsule Walls, 1-12 in *Encapsulation and Controlled Release*, Woodhead Publishing (1993). cited by applicant .

Fire & Xu, Rolling replication of short DNA circles, *PNAS* 92(10):4641-5 (1995). cited by applicant .

Firestine, S.M. et al., Using an AraC-based three hybrid system to detect biocatalysts in vivo, *Nat Biotechnol* 18: 544-547 (2000). cited by applicant .

Fisch et al., A strategy of exon shuffling for making large peptide repertoires displayed on filamentous bacteriophage, *PNAS* 93:7761-6 (1996). cited by applicant .

Fisher et al., Cell Encapsulation on a Microfluidic Platform, *The Eighth International Conference on Miniaturised Systems for Chemistry and Life Sciences, MicroTAS 2004*, Sep. 26-30, Malmo, Sweden. cited by applicant .

Fletcher et al., Micro reactors: principles and applications in organic synthesis, *Tetrahedron* 58:4735-4757 (2002). cited by applicant .

Fluri et al., Integrated capillary electrophoresis devices with an efficient postcolumn reactor in planar quartz and glass chips, *Anal Chem* 68:4285-4290 (1996). cited by applicant .

Formusek, L. et al., Polymeric microspheres as diagnostic tools for cell surface marker tracing, *Crit Rev Ther Drug Carrier Syst*, 2:137-74 (1986). cited by applicant .

Fowler, Enhancement of Mixing by Droplet-Based Microfluidics, *Int Conf MEMS* 97-100 (2002). cited by applicant .

Freese, E., The specific mutagenic effect of base analogues on Phage T4, *J Mol Biol*, 1: 87 (1959). cited by applicant .

Frenz et al., Reliable microfluidic on-chip incubation of droplets in delay-lines, *Lab on a Chip* 9:1344-1348 (2008). cited by applicant .

Fu et al., A microfabricated fluorescence-activated cell sorter, *Nature Biotechnology*, 17(11):1109-1111 (1999). cited by applicant .

Fu et al., An Integrated Microfabricated Cell Sorter, *Anal. Chem.*, 74: 2451-2457 (2002). cited by applicant .

Fulton et al., Advanced multiplexed analysis with the FlowMetrix system, *Clin Chem* 43:1749-1756 (1997). cited by applicant .

Fulwyler, Electronic Separation of Biological Cells by Volume, *Science* 150(3698):910-911 (1965). cited by applicant .

Gallarate et al., On the stability of ascorbic acid in emulsified systems for topical and cosmetic use,

- Int J Pharm 188(2):233-241 (1999). cited by applicant .
- Ganan-Calvo, A.M., Perfectly Monodisperse Microbubbling by Capillary Flow Focusing, *Phys Rev Lett* 87(27): 274501-1-4 (2001). cited by applicant .
- Ganan-Calvo, Generation of Steady Liquid Microthreads and Micron-Sized Monodisperse Sprays and Gas Streams, *Phys Rev Lett* 80(2):285-288 (1998). cited by applicant .
- Garcia-Ruiz et al. A super-saturation wave of protein crystallization, *J. Crystal Growth*, 232:149-155(2001). cited by applicant .
- Garcia-Ruiz et al, Investigation on protein crystal growth by the gel acupuncture method{, *Acta, Cryst.*, 1994, D50, 99. pp. 484-490. cited by applicant .
- Garstecki, et al., Formation of monodisperse bubbles in a microfluidic flow-focusing device, *Appl Phys Lett* 85(13):2649-2651 (2004). cited by applicant .
- Gasperlin et al., The structure elucidation of semisolid w/o emulsion systems containing silicone surfactant, *Intl J Pharm*, 107:51-6 (1994). cited by applicant .
- Gasperlin et al., Viscosity prediction of lipophilic semisolid emulsion systems by neural network modeling, *Intl J Pharm*, 196:37-50 (2000). cited by applicant .
- Georgiou et al., Display of heterologous proteins on the surface of microorganisms: from the screenign of combinatorial libraires to live recombinant vaccines. *Nat Biotechnol* 15(1), 29-34 (1997). cited by applicant .
- Georgiou, G., Analysis of large libraries of protein mutants using flow cytometry, *Adv Protein Chem*, 55: 293-315 (2000). cited by applicant .
- Gerds et al., A Synthetic Reaction NetWork: Chemical Amplification Using Nonequilibrium Autocatalytic Reactions Coupled in Time, *J. Am. Chem. Soc* 126:6327-6331 (2004). cited by applicant .
- Ghadessy et al., Directed Evolution of Polymerase Function by Compartmentalized Self-Replication, *PNSAS* 98(8): 4552-4557 (2001). cited by applicant .
- Gibbs et al., Detection of single DNA base differences by competitive oligonucleotide priming, *Nucleic Acids Res.* 17(7): 2437-48 (1989). cited by applicant .
- Gilliland, G., Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction, *PNAS*, 87(7):2725-9 (1990). cited by applicant .
- Giusti et al., Synthesis and characterization of 5' fluorescent dye labeled oligonucleotides, *Genome Res* 2:223-227 (1993). cited by applicant .
- Gold et al., Diversity of Oligonucleotide Functions *Annu Rev Biochem*, 64: 763-97 (1995). cited by applicant .
- Goodall, J. L. et al, Operation of Mixed-Culture Immobilized Cell Reactors for the Metabolism of Meta- and Para-Nitrobenzoate by *Comamonas* Sp. JS46 and *Comamonas* Sp. JS47, *Biotechnology and Bioengineering*, 59 (1): 21-27 (1998). cited by applicant .
- Gordon and Balasubramanian, Solid phase synthesis--designer linkers for combinatorial chemistry: a review, *J. Chem. Technol. Biotechnol.*, 74(9):835-851 (1999). cited by applicant .
- Grasland-Mongrain et al., Droplet coalescence in microfluidic devices, 30 pages (Jul. 2003) From internet: <http://www.eleves.ens.fr/home/grasland/rapports/stage4.pdf> cited by applicant .
- Green, R. and Szostak, J.W., Selection of a Ribozyme That Functions as a Superior Template in a Self Copying Reaction, *Science*, 258: 1910-5 (1992). cited by applicant .
- Gregoriadis, G., Enzyme entrapment in liposomes, *Methods Enzymol* 44:218-227 (1976). cited by applicant .
- Griffiths et al., Directed evolution of an extremely fast phosphotriesterase by in vitro

compartmentalization, *EMBO J*, 22:24-35 (2003). cited by applicant .

Griffiths et al., Isolation of high affinity human antibodies directly from large synthetic repertoires, *Embo J* 13(14):3245-60 (1994). cited by applicant .

Griffiths et al., Man-made enzymes-from design to in vitro compartmentalisation, *Curr Opin Biotechnol* 11:338-353 (2000). cited by applicant .

Griffiths, A., and Tawfik, D., Miniaturising the laboratory in emulsion droplets, *Trend Biotech* 24(9):395-402 (2006). cited by applicant .

Griffiths, A.D. et al., Strategies for selection of antibodies by phage display, *Curr Opin Biotechnol*, 9:102-8 (1998). cited by applicant .

Guatelli, J.C. et al., Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication, *PNAS*, 87(5):1874-8 (1990). cited by applicant .

Guixé et al., Ligand-Induced Conformational Transitions in *Escherichia coli* Phosphofructokinase 2: Evidence for an Allosteric Site for MgATP_{2n}, *Biochem.*, 37: 13269-12375 (1998). cited by applicant .

Gupta, K.C., et al., A general method for the synthesis of 3'-sulfhydryl and phosphate group containing oligonucleotides, *Nucl Acids Res* 19 (11): 3019-3026 (1991). cited by applicant .

Haber et al., Activity and spectroscopic properties of bovine liver catalase in sodium bis(2-ethylhexyl) sulfosuccinate/isooctane reverse micelles, *Eur J Biochem* 217(2): 567-73 (1993). cited by applicant .

Habig and Jakoby, Assays for differentiation of glutathione S-transferases, *Methods in Enzymology*, 77: 398-405 (1981). cited by applicant .

Hadd et al., Microchip Device for Performing Enzyme Assays, *Anal. Chem* 69(17): 3407-3412 (1997). cited by applicant .

Haddad et al., A methodology for solving physiologically based pharmacokinetic models without the use of simulation software, *Toxicol Lett.* 85(2): 113-26 (1996). cited by applicant .

Hagar and Spitzer, The effect of endotoxemia on concanavalin A induced alterations in cytoplasmic free calcium in rat spleen cells as determined with Fluo-3, *Cell Calcium* 13:123-130 (1992). cited by applicant .

Hai et al., Investigation on the release of fluorescent markers from the w/o/w emulsions by fluorescence-activated cell sorter, *J Control Release*, 96(3): 393-402 (2004). cited by applicant .

Haies et al., Morphometric study of rat lung cells. I. Numerical and dimensional characteristics of parenchymal cell population, *Am. Rev. Respir. Dis.* 123:533-54 (1981). cited by applicant .

Hall, Experimental evolution of Ebg enzyme provides clues about the evolution of catalysis and to evolutionary potential, *FEMS Microbiol Lett*, 174(1):1-8 (1999). cited by applicant .

Hall, The EBG system of *E. coli*: origin and evolution of a novel beta-galactosidase for the metabolism of lactose, *Genetica* 118(2-3):143-56 (2003). cited by applicant .

Han et al., Quantum-dot-tagged Microbeads for Multiplexed Optical Coding of Biomolecules, *Nat Biotech* 19(7): 631-635 (2001). cited by applicant .

Handen, J.S., High-throughput screening-challenges for the future, *Drug Discov World*, 47-50 (2002). cited by applicant .

Handique, K. et al., On-Chip Thermopneumatic Pressure for Discrete Drop Pumping, *Analytical Chemistry*, 73:1831-1838 (2001). cited by applicant .

Hanes et al., Degradation of porous poly(anhydride-co-imide) microspheres and implication for controlled macromolecule delivery, *Biomaterials*, 19(1-3): 163-172(1998). cited by applicant .

Hanes et al., In vitro selection and evolution of functional proteins by using ribosome display, *PNAS*

94:4937-42 (1997). cited by applicant .

Hansen et al., A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion, PNAS 99(26):16531-16536 (2002). cited by applicant .

Harada et al., Monoclonal antibody G6K12 specific for membrane-associated differentiation marker of human stratified squamous epithelia and squamous cell carcinoma, J. Oral Pathol. Med 22(4):145-152 (1993). cited by applicant .

Harder, K.W. et al., Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase beta (HPTO beta) using synthetic phosphopeptides, Biochem J 298 (Pt 2): 395-401 (1994). cited by applicant .

Harries et al., A Numerical Model for Segmented Flow in a Microreactor, Int J of Heat and Mass Transfer, 46:3313-3322 (2006). cited by applicant .

Harris et al., Single-molecule DNA sequencing of a viral genome, Science 320(5872):106-109 (2008). cited by applicant .

Harrison et al., Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip, Science 261(5123):895-897 (1993). cited by applicant .

Hasina et al., Plasminogen activator inhibitor-2: a molecular biomarker for head and neck cancer progression, Cancer Research 63:555-559 (2003). cited by applicant .

Haynes Principles of Digital PCR and Measurement Issue Oct. 15, 2012. cited by applicant .

Hayward et al., Dewetting Instability during the Formation of Polymersomes from BlockCopolymer-Stabilized Double Emulsions, Langmuir, 22(10): 4457-4461 (2006). cited by applicant .

He et al., Selective encapsulation of single cells and subcellular organelles into picoliter- and femtoliter-volume droplets, Anal Chem 77(6):1539-1544 (2005). cited by applicant .

Heim et al., Engineering Green Fluorescent Protein for Improved Brightness, Longer Wavelengths and Fluorescence Response Energy Transfer, Carr. Biol, 6(2): 178-182 (1996). cited by applicant .

Hellman et al., Differential tissue-specific protein markers of vaginal carcinoma, Br J Cancer, 100(8): 1303-131 (2009). cited by applicant .

Hergenrother et al., Small-Molecule Microarrays: Covalent Attachment and Screening of Alcohol-Containing Small Molecules on Glass Slides, J. Am. Chem. Soc, 122: 7849-7850 (2000). cited by applicant .

Hildebrand et al., Liquid-Liquid Solubility of Perfluoromethylcyclohexane with Benzene, Carbon Tetrachloride, Chlorobenzene, Chloroform and Toluene, J. Am. Chem. Soc, 71:22-25 (1949). cited by applicant .

Hjelmfelt et al., Pattern-Recognition in Coupled Chemical Kinetic Systems, Science, 260(5106):335-337 (1993). cited by applicant .

Ho, S.N. et al., Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene, 77(1):51-9 (1989). cited by applicant .

Hoang, Physiologically based pharmacokinetic models: mathematical fundamentals and simulation implementations, Toxicol Lett 79(1-3):99-106 (1995). cited by applicant .

Hochuli et al., New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues, J Chromatogr 411: 177-84 (1987). cited by applicant .

Holmes et al., Reagents for Combinatorial Organic Synthesis: Development of a New O-Nitrobenzyl Photolabile Linker for Solid Phase Synthesis, J. OrgChem., 60: 2318-2319(1995). cited by applicant .

Hong, S.B. et al., Stereochemical constraints on the substrate specificity of phosphodiesterase, Biochemistry, 38: 1159-1165 (1999). cited by applicant .

Hoogenboom et al., Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains, *Nucl Acids Res.*, 91:4133-4137 (1991). cited by applicant .

Hoogenboom, H.R., Designing and optimizing library selection strategies for generating high-affinity antibodies, *Trends Biotechnol*, 15:62-70 (1997). cited by applicant .

Hopfinger & Lasheras, Explosive Breakup of a Liquid Jet by a Swirling Coaxial Jet, *Physics of Fluids* 8(7):1696-1700 (1996). cited by applicant .

Hopman et al., Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for In situ hybridization using CARC amplification, *J of Histochem and Cytochem*, 46(6):771-77 (1998). cited by applicant .

Horton et al., Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension, *Gene* 77(1), 61-8 (1989). cited by applicant .

Hosokawa, Kazuo et al., Handling of Picoliter Liquid Samples in a Poly(dimethylsiloxane)-Based Microfluidic Device, *Analytical Chemistry*, 71(20):4781-4785 (1999). cited by applicant .

Hsu et al., Comparison of process parameters for microencapsulation of plasmid DNA in poly(D, L-lactic-co-glycolic acid microspheres, *J Drug Target*, 7:313-23 (1999). cited by applicant .

Huang L. R. et al., Continuous particle separation through deterministic lateral displacement, *Science* 304(5673):987-990 (2004). cited by applicant .

Huang, Z. et al., A sensitive competitive ELISA for 2,4-dinitrophenol using 3,6-fluorescein diphosphate as a fluorogenic substrate, *J Immunol Meth*, 149:261 (1992). cited by applicant .

Huang, Z.J., Kinetic assay of fluorescein mono-beta-D-galactosidase hydrolysis by beta-galactosidase: a front-face measurement for strongly absorbing fluorogenic substrates, *Biochemistry*, 30:8530-4 (1991). cited by applicant .

Hubert et al. Data Concordance from a Comparison between Filter Binding and Fluorescence Polarization Assay Formats for Identification of RUOck-II Inhibitors, *J biomol Screen* 8(4):399-409 (2003). cited by applicant .

Huebner, A. et al., Quantitative detection of protein expression in single cells using droplet microfluidics, *Chem Com* 12:1218-1220 (2007). cited by applicant .

Hung et al., Optimization of Droplet Generation by controlling PDMS Surface Hydrophobicity, 2004 ASME International Mechanical Engineering Congress and RD&D Expo, Nov. 13-19, Anaheim, CA (2004). cited by applicant .

Hung, et al, Controlled Droplet Fusion in Microfluidic Devices, *MicroTAS 2004*, Sep. 26-30, Malmo, Sweden (2004). cited by applicant .

Hutchison et al., Cell-free cloning using Phi29 polymerase, *PNAS* 102(48):17332-17336 (2005). cited by applicant .

Ibrahim, S.F. et al., High-speed cell sorting: fundamentals and recent advances, *Curr Opin Biotchnol*, 14(1):5-12 (2003). cited by applicant .

Ikeda et al., Bioactivation of tegafur to 5-fluorouracil is catalyzed by cytochrome P-450 2A6 in human liver microsomes in vitro, *Clin Cancer Res* 6(11):4409-4415 (2000). cited by applicant .

Inai et al., Immunohistochemical detection of an enamel protein-related epitope in rat bone at an early stage of osteogenesis, *Histochemistry* 99(5):335-362 (1993). cited by applicant .

International Preliminary Report of Patentability for PCTUS2010061741 Mailed Sep. 16, 2011 (4 pages). cited by applicant .

International Preliminary Report on Patentability mailed Sep. 20, 2007, for PCT/US2006/007772. cited by applicant .

International Search Report and Written Opinion for PCT/US2009/050931 Mailed Nov 26, 2009 (3 pages). cited by applicant .

International Search Report and Written Opinion for PCTUS1154353 Mailed Apr. 20, 2012 (34 pages.). cited by applicant .

International Search Report and Written Opinion for PCTUS12024745 Mailed May 11, 2012 (21 pages). cited by applicant .

International Search Report and Written Opinion for PCTUS1224741 Mailed Jun. 12, 2012 (12 pages). cited by applicant .

International Search Report and Written Opinion for PCTUS125499 Mailed May 29, 2012 (10 pages). cited by applicant .

International Search Report and Written Opinion in PCT/EP2010/065188 Mailed Jan. 12, 2011 (7 pages). cited by applicant .

International Search Report and Written Opinion in PCT/US11/24615 Mailed Jul. 25, 2011 (37 pages). cited by applicant .

International Search Report and Written Opinion in PCT/US2004/010903 Mailed Dec. 20, 2004 (16 pages). cited by applicant .

International Search Report and Written Opinion in PCT/US2006/021286 Mailed Sep. 14, 2007 (16 pages). cited by applicant .

International Search Report and Written Opinion in PCT/US2007/002063 Mailed Nov. 15, 2007 (20 pages). cited by applicant .

International Search Report in PCT/US01/18400 Mailed Jan. 28, 2005 (37 pages). cited by applicant .

Ismagilov, Integrated Microfluidic Systems, *Angew. Chem. Int. Ed* 42:4130-4132 (2003). cited by applicant .

Janda, et al, Chemical selection for catalysis in combinatorial antibody libraries, *Science*, 275:945-948 (1997). cited by applicant .

Jang et al., Controllable delivery of non-viral DNA from porous scaffold, *J Controlled Release* 86(1):157-168 (2003). cited by applicant .

Japanese Office Action for JP 2006-509830 mailed Jun. 1, 2011 (4 pages). cited by applicant .

Jermutus et al., Recent advances in producing and selecting functional proteins by using cell-free translation, *Curr Opin Biotechnol* 9(5): 534-48 (1998). cited by applicant .

Jestin et al., A Method for the Selection of Catalytic Activity Using Phage Display and Proximity Coupling, *Agnew. Chem. Int. Ed. Engi.* 38(8):1124-1127 (1999). cited by applicant .

Jo, et al, Encapsulation of Bovine Serum Albumin in Temperature-Programmed Shell-in-Shell Structures, *Macromol. Rapid Comm* 24:957-962 (2003). cited by applicant .

Joerger et al., Analyte detection with DNA-labeled antibodies and polymerase chain reaction, *Clin. Chem.* 41(9):1371-7 (1995). cited by applicant .

Johannsson et al., Amplification by Second Enzymes, In *ELISA and Other Solid Phase Immunoassays*, Kemeny et al (ed.), Chapter 4, pp. 85-106 John Wiley (1988). cited by applicant .

Johannsson, A., Heterogeneous Enzyme Immunoassays, In *Principles and Practice of Immunoassay*, pp. 295-325 Stockton Press (1991). cited by applicant .

Johnson, T.O. et al., Protein tyrosine phosphatase 1B inhibitors for diabetes, *Nature Review Drug Discovery* 1, 696-709 (2002). cited by applicant .

Jones et al. Glowing jellyfish, luminescence and a molecule called coelenterazine, *Trends Biotechnol.* 17(12):477-81 (1999). cited by applicant .

- Jones, L.J. et al., Quenched BODIPY dye-labeled casein substrates for the assay of protease activity by direct fluorescence measurement, *Anal Biochem*, 251:144 (1997). cited by applicant .
- Joo et al., Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation, *Nature* 399:670 (1999). cited by applicant .
- Joos et al., Covalent attachment of hybridizable oligonucleotides to glass supports, *Analytical Biochemistry* 247:96-101 (1997). cited by applicant .
- Joyce, G.F., In vitro Evolution of Nucleic Acids, *Curr. Opin. Structural Biol*, 4: 331-336 (1994). cited by applicant .
- Kadir and Moore, Haem binding to horse spleen ferritin, *Febs Lett*, 276: 81-4 (1990). cited by applicant .
- Kallen, R.G. et al., The mechanism of the condensation of formaldehyde with tetrahydrofolic acid, *J. Biol. Chem.*, 241:5851-63 (1966). cited by applicant .
- Kambara et al., Optimization of Parameters in a DNA Sequencer Using Fluorescence Detection, *Nature Biotechnology* 6:816-821 (1988). cited by applicant .
- Kamensky et al., Spectrophotometer: new instrument for ultrarapid cell analysis, *Science* 150(3696):630-631 (1965). cited by applicant .
- Kanouni et al., Preparation of a stable double emulsion (W1/O/W2): role of the interfacial films on the stability of the system, *Adv. Collid. Interf. Sci.*, 99(3): 229-254 (2002). cited by applicant .
- Katanaev et al., Viral Q beta RNA as a high expression vector for mRNA translation in a cell-free system, *Febs Lett*, 359:89-92 (1995). cited by applicant .
- Katsura et al., Indirect micromanipulation of single molecules in water-in-oil emulsion, *Electrophoresis*, 22:289-93 (2001). cited by applicant .
- Kawakatsu et al., Regular-sized cell creation in microchannel emulsification by visual microprocessing method, *Journal of the American Oil Chemists Society*, 74:317-21 (1997). cited by applicant .
- Keana J. & Cai, S. X., New reagents for photoaffinity labeling: synthesis and photolysis of functionalized perfluorophenyl azides, *J. Org. Chem.* 55(11):3640-3647 (1990). cited by applicant .
- Keefe, A.D. et al., Functional proteins from a random-sequence library, *Nature*, 410: 715-718 (2001). cited by applicant .
- Keij et al., High-Speed Photodamage Cell Selection Using a Frequency-Doubled Argon Ion Laser, *Cytometry*, 19(3): 209-216 (1995). cited by applicant .
- Keij, J.F., et al., High-speed photodamage cell sorting: An evaluation of the ZAPPER prototype, *Methods in cell biology*, 42: 371-358 (1994). cited by applicant .
- Kelly et al., Miniaturizing chemistry and biology in microdroplets, *Chem Commun* 18:1773-1788 (2007). cited by applicant .
- Kerker, M., Elastic and inelastic light scattering in flow cytometry, *Cytometry*, 4:1-10 (1983). cited by applicant .
- Khandjian, UV crosslinking of RNA to nylon membrane enhances hybridization signals, *Mol. Bio. Rep.* 11: 107-115 (1986). cited by applicant .
- Kim et al., Comparative study on sustained release of human growth hormone from semi-crystalline poly(L-lactic acid) and amorphous poly(D,L-lactic-co-glycolic acid) microspheres: morphological effect on protein release, *Journal of Controlled Release*, 98(1):115-125 (2004). cited by applicant .
- Kim S. et al, Type II quantum dots: CdTe/CdSe (core/shell) and CdSe/ZnTe (core/shell) heterostructures, *J. Am Chem Soc.* 125:11466-11467 (2003). cited by applicant .
- Kircher et al., High-throughput DNA sequencing-concepts and limitations, *Bioessays* 32(6):524-

- 536 (2010). cited by applicant .
- Kiss et al., High-throughput quantitative polymerase chain reaction in picoliter droplets, *Anal. Chem* 80:8975-8981 (2008). cited by applicant .
- Kitagawa et al., Manipulation of a single cell with microcapillary tubing based on its electrophoretic mobility, *Electrophoresis* 16:1364-1368 (1995). cited by applicant .
- Klug and Famulok, All you wanted to know about selex, *Molecular Biology Reports*, 20:97-107 (1994). cited by applicant .
- Klug and Schwabe, Protein motifs 5. Zinc fingers, *FASEB J* 9(8):597-604 (1995). cited by applicant .
- Klug, A., Gene Regulatory Proteins and Their Interaction with DNA, *Ann NY Acad Sci*, 758: 143-60 (1995). cited by applicant .
- Knaak et al., Development of partition coefficients, V_{max} and K_m values, and allometric relationships, *Toxicol Lett.* 79(1-3):87-98 (1995). cited by applicant .
- Knight, James B., Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds, *Physical Review Lett* 80(17):3863-3866 (1998). cited by applicant .
- Kojima et al. PCR amplification from single DNA molecules on magnetic beads in emulsion: application for high-throughput screening of transcription factor targets. *Nucleic Acids Res.* 33:e150 (2005). cited by applicant .
- Kolb et al., Cotranslational folding of proteins, *Biochem Cell Biol*, 73:1217-20 (1995). cited by applicant .
- Komatsu et al., Roles of cytochromes P450 1A2, 2A6, and 2C8 in 5-fluorouracil formation from tegafur, an anticancer prodrug, in human liver microsomes. *Drug Met. Disp.*, 28:1457-1463 (2001). cited by applicant .
- Kopp et al., Chemical amplification: continuous flow PCR on a chip, *Science*, 280:1046-48 (1998). cited by applicant .
- Koster et al., Drop-based microfluidic devices for encapsulation of single cells, *Lab on a Chip* 8:1110-1115 (2008). cited by applicant .
- Kowalczykowski et al., Biochemistry of homologous recombination in *Escherichia coli*, *Microbiol Rev* 58(3):401-65 (1994). cited by applicant .
- Krafft et al., Emulsions and microemulsions with a fluorocarbon phase, *Colloid and Interface Science* 8(3):251-258 (2003). cited by applicant .
- Krafft, Fluorocarbons and fluorinated amphiphiles in drug delivery and biomedical research, *Adv Rev Drug Disc* 47:209-228 (2001). cited by applicant .
- Krafft et al., Synthesis and preliminary data on the biocompatibility and emulsifying properties of perfluoroalkylated phosphoramidates as injectable surfactants, *Eur. J. Med. Chem.*, 26:545-550 (1991). cited by applicant .
- Kralj et al., Surfactant-enhanced liquid-liquid extraction in microfluidic channels with inline electric-field enhanced coalescence, *Lab Chip* 5:531-535 (2005). cited by applicant .
- Kricka and Wilding, Microchip PCR, *Anal Bioanal Chem* 377(5):820-825 (2003). cited by applicant .
- Kricka and Wilding, Micromachining: a new direction for clinical analyzers, *Pure and Applied Chemistry* 68(10):1831-1836 (1996). cited by applicant .
- Krumdiek, C.L. et al., Solid-phase synthesis of pteroylpolylglutamates, *Methods Enzymol*, 524-29 (1980). cited by applicant .
- Kumar, A. et al., Activity and kinetic characteristics of glutathione reductase in vitro in reverse

- micellar waterpool, *Biochem Biophys Acta*, 996(1-2):1-6 (1989). cited by applicant .
- Lage et al., Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. *Genome Res.* 13: 294-307 (2003). cited by applicant .
- Lamprecht et al., pH-sensitive microsphere delivery increases oral bioavailability of calcitonin, *Journal of Controlled Release*, 98(1): 1-9(2004). cited by applicant .
- Lancet, D. et al., Probability model for molecular recognition in biological receptor repertoires: significance to the olfactory system, *PNAS*, 90(8):3715-9 (1993). cited by applicant .
- Landergren et al., A ligase mediated gene detection technique. *Science* 241(4869):1077-80 (1988). cited by applicant .
- Lasheras, et al., Breakup and Atomization of a Round Water Jet by a High Speed Annular Air Jet, *J Fluid Mechanics* 357:351-379 (1998). cited by applicant .
- Leary et al., Application of Advanced Cytometric and Molecular Technologies to Minimal Residual Disease Monitoring, *Proceedings of SPIE* 3913:36-44 (2000). cited by applicant .
- Lee et al, Investigating the target recognition of DNA cytosine-5 methyltransferase HhaI by library selection using in vitro compartmentalisation (IVC), *Nucleic Acids Res* 30:4937-4944 (2002). cited by applicant .
- Lee et al., Circulating flows inside a drop under time-periodic non-uniform electric fields, *Phys Fluids* 12(8):1899-1910 (2000). cited by applicant .
- Lee, et al, Effective Formation of Silicone-in-Fluorocarbon-in-Water Double Emulsions: Studies on Droplet Morphology and Stability, *Journal of Dispersion Sci Tech* 23(4):491-497(2002). cited by applicant .
- Lee, et al, Preparation of Silica Particles Encapsulating Retinol Using O/W/O Multiple Emulsions, *Journal of Colloid and Interface Science*, 240(1): 83-89 (2001). cited by applicant .
- Lemof, et al, An AC Magnetohydrodynamic Microfluidic Switch for Micro Total Analysis Systems, *Biomedical Microdevices*, 5(1):55-60 (2003). cited by applicant .
- Lesley et al., Use of in vitro protein synthesis from PCR-generated templates to study interaction of E coli transcription factors with core RNA polymerase, *J Biol Chem* 266(4):2632-8 (1991). cited by applicant .
- Lesley, S.A., Preparation and use of E. coli S-30 extracts, *Methods Mol Biol*, 37:265-78 (1995). cited by applicant .
- Leung et al., A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 1:11-15 (1989). cited by applicant .
- Li and Harrison, Transport, Manipulation, and Reaction of Biological Cells On-Chip Using Electrokinetic Effects, *Analytical Chemistry* 69(8):1564-1568 (1997). cited by applicant .
- Li et al., Nanoliter microfluidic hybrid method for simultaneous screening and optimization validated with crystallization of membrane proteins, *PNAS* 103: 19243-19248 (2006). cited by applicant .
- Li et al., Single-step procedure for labeling DNA strand breaks with fluorescein-or BODIPY-conjugated deoxynucleotides: detection of apoptosis and bromodeoxyuridine incorporation. *Cytometry* 20:172-180 (1995). cited by applicant .
- Liao et al., Isolation of a thermostable enzyme variant by cloning and selection in a thermophile, *PNAS* 83:576-80 (1986). cited by applicant .
- Lim et al., Microencapsulated islets as bioartificial endocrine pancreas, *Science* 210(4472):908-10 (1980). cited by applicant .
- Link et al, Geometrically Mediated Breakup of Drops in Microfluidic Devices, *Phys. Rev. Lett.*,

- <http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnethtml%2FPTO%2Fsearch-bool.html&r=1&f=G&l=50&co1=AND&d...>

Dynabeads, and the characteristics of the bound nucleic acids in hybridization reactions, *Nucleic Acids Research*, Oxford University Press, 16(22) (1998). cited by applicant .

Lunderberg et al., Solid-phase technology: magnetic beads to improve nucleic acid detection and analysis, *Biotechnology Annual Review*, 1:373-401 (1995). cited by applicant .

Lundstrom, et al, Breakthrough in cancer therapy: Encapsulation of drugs and viruses, www.currentdrugdiscovery.com, Nov. 19-23, 2002. cited by applicant .

Lyne, P.D., Structure-Based Virtual Screening: An Overview, *Drug Discov. Today*, 7(20):1047-1055 (2002). cited by applicant .

Ma, C. et al, In vitro protein engineering using synthetic tRNA(Ala) with different anticodons, *Biochemistry* 32(31):7939-45 (1993). cited by applicant .

Mackenzie et al., The application of flow microfluorimetry to biomedical research and diagnosis: a review, *Dev Biol Stand* 64:181-193 (1986). cited by applicant .

Mackenzie, IABS Symposium on Reduction of Animal Usage in the Development and Control of Biological Products, London, UK, 1985. cited by applicant .

Maclean, D. et al., Glossary of terms used in combinatorial chemistry, *Pure Appl. Chem* 71(12):2349-2365 (1999). cited by applicant .

Magdassi et al., Multiple Emulsions: HLB Shift Caused by Emulsifier Migration to External Interface, *J. Colloid Interface Sci* 97:374-379 (1984). cited by applicant .

Mahajan et al., Bcl-2 and Bax Interactions in Mitochondria Probed with Green Florescent Protein and Fluorescence Resonance Energy Transfer, *Nat. Biotechnol.* 16(6): 547-552 (1998). cited by applicant .

Manley et al., In vitro transcription: whole cell extract, *Methods Enzymol*, 101:568-82 (1983). cited by applicant .

Manz et al., Micromachining of monocrystalline silicon and glass for chemical analysis systems A look into next century's technology or just a fashionable craze, *Trends in Analytical Chemistry* 10(5):144-149 (1991). cited by applicant .

Mao et al., Kinetic behaviour of alpha-chymotrypsin in reverse micelles: a stopped-flow study, *Eur J Biochem* 208(1):165-70 (1992). cited by applicant .

Mao, Q. et al., Substrate effects on the enzymatic activity of alphachymotrypsin in reverse micelles, *Biochem Biophys Res Commun*, 178(3):1105-12 (1991). cited by applicant .

Mardis, E.R., The impact of next-generation sequencing technology on genetics, *Trends Genet* 24:133-141 (2008). cited by applicant .

Margulies, M et al., Genome sequencing in microfabricated high-density picolitre reactors, *Nature* 437(7057):376-380 (2005). cited by applicant .

Marques et al., Porous Flow within Concentric Cylinders, *Bull Am Phys Soc Div Fluid Dyn* 41:1768 (1996). cited by applicant .

Mason, T.J. and Bibette, J. Shear Rupturing of Droplets in Complex Fluids, *Langmuir*, 13(17):4600-4613 (1997). cited by applicant .

Mastrobattista et al., High-throughput screening of enzyme libraries: in vitro evolution of a beta-galactosidase by fluorescence-activated sorting of double emulsions, *Chem. Biol.* 12(12): 1291-1300 (2005). cited by applicant .

Masui et al., Probing of DNA-Binding Sites of Escherichia coli RecA Protein Utilizing 1-anilinonaphthalene-8-Sulfonic Acid, *Biochem* 37(35):12133-12143 (1998). cited by applicant .

Matayoshi, E.D. et al., Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer, *Science* 247:954 (1990). cited by applicant .

- Mattheakis et al., An in vitro polysome display system for identifying ligands from very large peptide libraries, PNAS 91:9022-6 (1994). cited by applicant .
- Mayr, L.M., and Fuerst, P., The Future of High-Throughput Screening, J Biomol Screen 13:443-448 (2008). cited by applicant .
- Mazutis et al., Droplet-Based Microfluidic Systems for High-Throughput Single DNA Molecule Isothermal Amplification and Analysis, Anal Chem 81(12):4813-4821 (2009). cited by applicant .
- Mazutis et al., Multi-step microfluidic droplet processing: kinetic analysis of an in vitro translated enzyme, Lab Chip 9:2902-2908 (2009). cited by applicant .
- McCafferty et al., Phage antibodies: filamentous phage displaying antibody variable domains, Nature, 348: 552-4 (1990). cited by applicant .
- McDonald and Whitesides, Poly(dimethylsiloxane) as a material for fabricating microfluidic devices, Account Chem. Res. 35:491-499 (2002). cited by applicant .
- McDonald et al. Fabrication of microfluidic systems in poly(dimethylsiloxane), Electrophoresis 21(1):27-40 (2000). cited by applicant .
- Melton et al., Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter, Nucl. Acids Res. 12(18):7035-7056 (1984). cited by applicant .
- Mendel, D. et al., Site-Directed Mutagenesis with an Expanded Genetic Code, Annu Rev Biophys Biomol Struct, 24:435-62 (1995). cited by applicant .
- Menger and Yamada, Enzyme catalysis in water pools, J. Am. Chem. Soc., 101:6731-4 (1979). cited by applicant .
- Meylan and Howard, Atom/fragment contribution method for estimating octanol-water partition coefficients, J Pharm Sci. 84(1):83-92 (1995). cited by applicant .
- Miele et al., Autocatalytic replication of a recombinant RNA, J Mol Biol, 171:281-95 (1983). cited by applicant .
- Minshuil, J. and Stemmer, W.P., Protein evolution by molecular breeding, Curr Opin Chem Biol 3(3): 284-90 (1999). cited by applicant .
- Miroux and Walker, Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, J of Mol Biol 260(3):289-98 (1996). cited by applicant .
- Miyawaki et al., Fluorescent Indicators for Ca²⁺ Based on Green Fluorescent Proteins and Calmodulin, Nature, 388: 882-887 (1997). cited by applicant .
- Mize et al., Dual-enzyme cascade--an amplified method for the detection of alkaline phosphatase, Anal Biochem 179(2): 229-35 (1989). cited by applicant .
- Mock et al., A fluorometric assay for the biotin-avidin interaction based on displacement of the fluorescent probe 2-anilino-naphthalene-6-sulfonic acid, Anal Biochem, 151:178-81 (1985). cited by applicant .
- Moldavan, A., Photo-electric technique for the counting of microscopical cells, Science 80:188-189 (1934). cited by applicant .
- Montigiani, S. et al., Alanine substitutions in calmodulin-binding peptides result in unexpected affinity enhancement, J Mol Biol, 258:6-13 (1996). cited by applicant .
- Moore, M.J., Exploration by lamp light, Nature, 374:766-7 (1995). cited by applicant .
- Moudrianakis and Beer, Base sequence determination in nucleic acids with the electron microscope 3. Chemistry and microscopy of guanine-labeled DNA, PNAS 53:564-71 (1965). cited by applicant .

- Mueth et al., Origin of stratification in creaming emulsions, *Physical Review Letters* 77(3):578-581 (1996). cited by applicant .
- Mulbry, W.W. et al., Parathion hydrolase specified by the *Flavobacterium opd* gene: relationship between the gene and protein. *J Bacteriol*, 171: 6740-6746 (1989). cited by applicant .
- Mulder et al., Characterization of two human monoclonal antibodies reactive with HLA-B12 and HLA-B60, respectively, raised by in vitro secondary immunization of peripheral blood lymphocytes, *Hum. Immunol* 36(3):186-192 (1993). cited by applicant .
- Nakano et al., High speed polymerase chain reaction in constant flow, *Biosci Biotech and Biochem*, 58:349-52 (1994). cited by applicant .
- Nakano et al., Single-molecule PCR using water-in-oil emulsion, *J Biotech*, 102:117-24 (2003). cited by applicant .
- Nakano et al., Single-molecule reverse transcription polymerase chain reaction using water-in-oil emulsion, *J Biosci Bioeng* 99:293-295 (2005). cited by applicant .
- Nametkin, S.N. et al., Cell-free translation in reversed micelles, *FEB Letters*, 309(3):330-32 (1992). cited by applicant .
- Narang et al, Improved phosphotriester method for the synthesis of gene fragments, *Methods Enzymol*, 68:90-98 (1979). cited by applicant .
- Nelson, P. S., et al, Bifunctional oligonucleotide probes synthesized using a novel CPG support are able to detect single base pair mutations, *Nucl Acids Res* 17(18): 7187-7194 (1989). cited by applicant .
- Nemoto et al., In vitro virus: bonding of mRNA bearing puromycin at the 3 terminal end to the C-terminal end of its encoded protein on the ribosome in vitro, *Federation of European Biochemical Societies*, 414:405-8 (1997). cited by applicant .
- Ness, J.E. et al., Molecular Breeding: the natural approach to protein design. *Adv Protein Chem*, 55: 261-292 (2000). cited by applicant .
- Ng et al., Protein crystallization by capillary counter-diffusion for applied crystallographic structure determination, *J. Struct. Biol*, 142:218-231(2003). cited by applicant .
- Ng, B.L. et al., Factors affecting flow karyotype resolution, *Cytometry, Part A* 69A: 1028-1036 (2006). cited by applicant .
- Nguyen et al., Optical detection for droplet size control in microfluidic droplet-based analysis systems, *Sensors and Actuators B* 117(2):431-436 (2006). cited by applicant .
- Nihant et al., Polylactide Microparticles Prepared by Double Emulsion/Evaporation Technique. I. Effect of Primary Emulsion Stability, *Pharmaceutical Research*, 11(10):1479-1484 (1994). cited by applicant .
- Nisisako et al., Controlled formulation of monodisperse double emulsions in a multiple-phase microfluidic system, *Sot Matter*, 1:23-27 (2005). cited by applicant .
- Nisisako et al., Formation of droplets using branch channels in a microfluidic circuit, *Proceedings of the SICE Annual Conference. International Session Papers*, 1262-1264 (2002). cited by applicant .
- Nisisako et al., Microstructured Devices for Preparing Controlled Multiple Emulsions. *Chem. Eng. Technol* 31(8):1091-1098 (2008). cited by applicant .
- Nisisako, Takasi et al., Droplet Formation in a MicroChannel NetWORK, *Lab on a Chip*, vol. 2, 2002, pp. 24-26. cited by applicant .
- Nissim, A. et al., Antibody fragments from a single phage display library as immunochemical reagents, *Embo J*, 13:692-8 (1994). cited by applicant .
- Nof and Shea, Drug-releasing scaffolds fabricated from drug-loaded microspheres, *J. Biomed*

- Mater Res 59:349-356 (2002). cited by applicant .
- Norman, A., Flow Cytometry, Med. Phys., 7(6):609-615 (1980). cited by applicant .
- Oberholzer et al., Enzymatic RNA replication in self-reproducing vesicles: an approach to a minimal cell, Biochem Biophys Res Commun 207(1):250-7 (1995). cited by applicant .
- Oberholzer et al., Polymerase chain reaction in liposomes, Chem. Biol. 2(10):677-82 (1995). cited by applicant .
- Obukowicz, M.G. et al., Secretion and export of IGF-1 in *Escherichia coli* strain JM101, Mol Gen Genet, 215:19-25 (1988). cited by applicant .
- Office Action for U.S. Appl. No. 11/246,911 mailed Feb. 8, 2011. cited by applicant .
- Office Action for U.S. Appl. No. 11/360,845 mailed Apr. 26, 2011. cited by applicant .
- Office Action for U.S. Appl. No. 11/360,845 mailed Aug. 4, 2010. cited by applicant .
- Office Action for U.S. Appl. No. 11/698,298, mailed Jun. 29, 2011. cited by applicant .
- Ogura, Y., Catalase activity at high concentrations of hydrogen peroxide, Archs Biochem Biophys, 57: 288-300 (1955). cited by applicant .
- Oh et al., Distribution of Macropores in Silica Particles Prepared by Using Multiple Emulsions, Journal of Colloid and Interface Science, 254(1): 79-86 (2002). cited by applicant .
- Okushima et al. Controlled production of monodisperse double emulsions by two-step droplet breakup in microfluidic devices, Langmuir 20(23): 9905-8 (2004). cited by applicant .
- Olsen et al., Function-based isolation of novel enzymes from a large library, Nat Biotechnol 13(10):1071-4 (2000). cited by applicant .
- Omburo, G.A. et al., Characterization of the zinc binding site of bacterial phosphotriesterase, J of Biological Chem, 267:13278-83 (1992). cited by applicant .
- Oroskar et al., Detection of immobilized amplicons by ELISA-like techniques, Clin. Chem 42:1547-1555 (1996). cited by applicant .
- Ostermeier, M. et al., A combinatorial approach to hybrid enzymes independent of DNA homology, Nat Biotechnol, 17(12):1205-9 (1999). cited by applicant .
- Ouellette, A new wave of microfluidic devices, Indust Physicist pp. 14-17 (2003). cited by applicant .
- Pabit et al., Laminar-Flow Fluid Mixer for Fast Fluorescence Kinetics Studies, Biophys J 83:2872-2878 (2002). cited by applicant .
- Paddison et al., Stable suppression of gene expression by RNAi in mammalian cells, PNAS 99(3):1443-1448 (2002). cited by applicant .
- Pannacci et al., Equilibrium and Nonequilibrium States in Microfluidic Double Emulsions Physical Review Letters, 101(16):164502 (2008). cited by applicant .
- Park et al., Cylindrical compact thermal-cycling device for continuous flow polymerase chain reaction, Anal Chem, ACS, 75:6029-33 (2003). cited by applicant .
- Park et al., Model of Formation of Monodispersed Colloids, J. Phys. Chem. B 105:11630-11635 (2001). cited by applicant .
- Parker et al., Development of high throughput screening assays using fluorescence polarization: nuclear receptor-ligand-binding and kinase/phosphatase assays, J Biomol Screen, 5(2): 77-88 (2000). cited by applicant .
- Parmley et al., Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73(2):305-18 (1988). cited by applicant .
- Pedersen et al., A method for directed evolution and functional cloning of enzymes, PNAS 95(18):10523-8 (1998). cited by applicant .

- Pelham and Jackson, An efficient mRNA-dependent translation system from reticulocyte lysates, *Eur J Biochem* 67:247-56 (1976). cited by applicant .
- Pelletier et al., An in vivo library-verslibrary selection of optimized protein-protein interactions, *Nature Biotechnology*, 17:683-90 (1999). cited by applicant .
- Peng et al., Controlled Production of Emulsions Using a Crossflow Membrane, *Particle & Particle Systems Characterization* 15:21-25 (1998). cited by applicant .
- Perelson et al., Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. *J Theor Biol* 81(4):645-70 (1979). cited by applicant .
- Perez-Gilabert et al., Application of active-phase plot to the kinetic analysis of lipoxygenase in reverse micelles, *Biochemistry J.* 288:1011-1015 (1992). cited by applicant .
- Perrin, J., Polarisation de la lumiere de fluorescence vie moyenne des molecules dans letat excite, *J. Phys. Rad.* 1:390-401 (1926). cited by applicant .
- Petrounia, I.P. et al., Designed evolution of enzymatic properties, *Curr Opin Biotechnol*, 11:325-330 (2000). cited by applicant .
- Piemi et al., Transdermal delivery of glucose through hairless rat skin in vitro: effect of multiple and simple emulsions, *Int J Pharm*, 171:207-215 (1998). cited by applicant .
- Pirrung et al., A General Method for the Spatially Defined Immobilization of Biomolecules on Glass Surfaces Using 'Caged' Biotin, *Bioconjug Chem* 7: 317-321 (1996). cited by applicant .
- Ploem, in *Fluorescent and Luminescent Probes for Biological Activity* Mason, T. G. Ed., Academic Press, Landon, pp. 1-11, 1993. cited by applicant .
- Pluckthun, A. et al., In vitro selection and evolution of proteins, *Adv Protein Chem*, 55: 367-403 (2000). cited by applicant .
- Pollack et al., Electrowetting-based actuation of droplets for integrated microfluidics, *Lab Chip* 2:96-101 (2002). cited by applicant .
- Pollack et al., Selective chemical catalysis by an antibody, *Science* 234(4783):1570-3 (1986). cited by applicant .
- Pons et al, Synthesis of Near-Infrared-Emitting, Water-Soluble CdTeSe/CdZnS Core/Shell Quantum Dots, *Chemistry of Materials* 21(8):1418-1424 (2009). cited by applicant .
- Posner et al., Engineering specificity for folate into dihydrofolate reductase from *Escherichia coli*, *Biochemistry*, 35: 1653-63 (1996). cited by applicant .
- Poulin and Theil, "A priori" prediction of tissue: plasma partition coefficients of drugs to facilitate the use of physiologically-based pharmacokinetic models in drug discovery, *J Pharm Sci* 89(1):16-35 (2000). cited by applicant .
- Priest, et al. Generation of Monodisperse Gel Emulsions in a Microfluidic Device, *Applied Physics Letters*, 88:024106 (2006). cited by applicant .
- Qi et al., Acid Beta-Glucosidase: Intrinsic Fluorescence and Conformational Changes Induced by Phospholipids and Saposin C, *Biochem.*, 37(33): 11544-11554 (1998). cited by applicant .
- Raghuraman et al., Emulston Liquid Membranes for Wastewater Treatment: Equilibrium Models for Some Typical Metal-Extractant Systems, *Environ. Sci. Technol* 28:1090-1098 (1994). cited by applicant .
- Ralhan, Discovery and Verification of Head-and-neck Cancer Biomarkers by Differential Protein Expression Analysis Using iTRAQ Labeling, Multidimensional Liquid Chromatography, and Tandem Mass Spectrometry, *Mol Cell Proteomics* 7(6):1162-1173 (2008). cited by applicant .
- Ramsey, J.M., The burgeoning power of the shrinking laboratory, *Nat Biotechnol* 17(11):1061-2 (1999). cited by applicant .

- Ramstrom and Lehn, Drug discovery by dynamic combinatorial libraries, *Nat Rev Drug Discov* 1:26-36 (2002). cited by applicant .
- Raushel, F.M. et al, Phosphotriesterase: an enzyme in search of its natural substrate, *Adv Enzymol Relat Areas Mol Biol*, 74: 51-93 (2000). cited by applicant .
- Rech et al, Introduction of a yeast artificial chromosome vector into *Sarrachomyces cervesia* by electroporation, *Nucleic Acids Res* 18:1313 (1990). cited by applicant .
- Reyes et al, Micro Total Analysis Systems. 1. Introduction, Theory and Technology, *Anal Chem* 74(12):2623-2636 (2002). cited by applicant .
- Riess, J.S., Fluorous micro- and nanophases with a biomedical perspective, *Tetrahedron* 58(20):4113-4131 (2002). cited by applicant .
- Roach et al, Controlling nonspecific protein adsorption in a plug-based microfluidic system by controlling interfacial chemistry using fluorophase surfactants, *Anal. Chem.* 77:785-796 (2005). cited by applicant .
- Roberts & Ja, In vitro selection of nucleic acids and proteins: What are we learning, *Curr Opin Struct Biol* 9(4): 521-9 (1999). cited by applicant .
- Roberts et al, Simian virus 40 DNA directs synthesis of authentic viral polypeptides in a linked transcription-translation cell-free system 72(5):1922-1926 (1975). cited by applicant .
- Roberts, et al, RNA-peptide fusion for the in vitro selection of peptides and proteins, *PNAS* 94:12297-302 (1997). cited by applicant .
- Roberts, J.W., Termination factor for RNA synthesis, *Nature*, 224: 1168-74 (1969). cited by applicant .
- Roberts, R.W. Totally in vitro protein selection using mRNA-protein fusions and ribosome display. *Curr Opin Chem Biol* 3(3), 268-73 (1999). cited by applicant .
- Rodriguez-Antona et al, Quantitative RT-PCR measurement of human cytochrome P-450s: application to drug induction studies. *Arch. Biochem. Biophys.*, 376:109-116 (2000). cited by applicant .
- Rolland et al, Fluorescence Polarization Assay by Flow Cytometry, *J. Immunol. Meth.*, 76(1): 1-10 (1985). cited by applicant .
- Rosenberg et al, Termination of transcription in bacteriophage lambda, *J Biol Chem*, 250: 4755-64 (1975). cited by applicant .
- Rosenberry, T.L., Acetylcholinesterase, *Adv Enzymol Relat Areas Mol Biol*, 43: 103-218 (1975). cited by applicant .
- Rotman, Measurement of activities of single molecules of beta-galactosidase, *PNAS*, 47:1981-91 (1961). cited by applicant .
- Russon et al, Single-nucleotide polymorphism analysis by allele-specific extension of fluorescently labeled nucleotides in a microfluidic flow-through device, *Electrophoresis*, 24:158-61 (2003). cited by applicant .
- Sadtler et al, Achieving stable, reverse water-in-fluorocarbon emulsions. *Angew Chem Int Ed* 35:1976-1978 (1996). cited by applicant .
- Saiki, R.K., et al, Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239(4839):487-91 (1988). cited by applicant .
- Sakamoto, Rapid and simple quantification of bacterial cells by using a microfluidic device, *Appl Env Microb.* 71:2 (2005). cited by applicant .
- Sanchez et al, Breakup of Charged Capillary Jets, *Bulletin of the American Physical Society Division of Fluid Dynamics* 41:1768-1768 (1996). cited by applicant .

- Sano, T. et al., Immuno-PCR-Very sensitive antigen-detection by means of sepcific antibody-DNA conjugates. *Science* 258(5079), 120-122 (1992). cited by applicant .
- SantaLucia, A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics, *PNAS* 95(4):1460-5 (1998). cited by applicant .
- Santra et al., Fluorescence lifetime measurements to determine the core-shell nanostructure of FITC-doped silica nanoparticles: An optical approach to evaluate nanoparticle photostability, *J Luminescence* 117(1):75-82 (2006). cited by applicant .
- Schatz et al., Screening of peptide libraries linked to lac repressor, *Methods Enzymol* 267: 171-91 (1996). cited by applicant .
- Schneegass et al., Miniaturized flow-through PCR with different template types in a silicone chip thermocycler, *Lab on a Chip, Royal Soc of Chem*, 1:42-9 (2001). cited by applicant .
- Schubert et al., Designer Capsules, *Nat Med* 8:1362 (2002). cited by applicant .
- Schweitzer et al., Immunoassays with rolling circle DNA amplification: A versatile platform for ultrasensitive antigen detection, *PNAS* 97(18), 10113-10119 (2000). cited by applicant .
- Schweitzer, B. et al., Combining nucleic acid amplification and detection. *Curr Opin Biotechnol* 12(1):21-7 (2001). cited by applicant .
- Scott, R.L., The Solubility of Fluorocarbons, *J. Am. Chem. Soc.*, 70: 4090-4093 (1948). cited by applicant .
- Seethala and Menzel, Homogeneous, Fluorescence Polarization Assay for Src-Family Tyrosine Kinases, *Anal Biochem* 253(2):210-218 (1997). cited by applicant .
- Seiler et al., Planar glass chips for capillary electrophoresis: repetitive sample injection, quantitation, and separation efficiency, *Anal Chem* 65(10):1481-1488 (1993). cited by applicant .
- Selwyn M. J., A simple test for inactivation of an enzyme during assay, *Biochim Biophys Acta* 105:193-195 (1965). cited by applicant .
- Seo et al., Microfluidic consecutive flow-focusing droplet generators, *Soft Matter*, 3:986-992 (2007). cited by applicant .
- Seong and Crooks, Efficient Mixing and Reactions Within Microfluidic Channels Using Microbead-Supported Catalysts, *J Am Chem Soc* 124(45):13360-1 (2002). cited by applicant .
- Seong et al., Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs Within Microfluidic Systems: Application to DNA Hybridization, *Analytical Chem* 74(14):3372-3377 (2002). cited by applicant .
- Sepp et al., Microbead display by in vitro compartmentalisation: selection for binding using flow cytometry, *FEBS Letters* 532:455-58 (2002). cited by applicant .
- Serpensu et al., Reversible and irreversible modification of erythrocyte membrane permeability by electric field, *Biochim Biophys Acta* 812(3):779-785 (1985). cited by applicant .
- Shapiro, H.M., Multistation multiparameter flow cytometry: a critical review and rationale, *Cytometry* 3: 227-243 (1983). cited by applicant .
- Shestopalov et al., Multi-Step Synthesis of Nanoparticles Performed on Millisecond Time Scale in a Microfluidic Droplet-Based System, *The Royal Society of Chemistry* 4:316-321(2004). cited by applicant .
- Shtern V, and Hussain F., Hysteresis in swirling jets, *J. Fluid Mech.* 309:1-44 (1996). cited by applicant .
- Sia & Whitesides, Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies, *Electrophoresis* 24(21):3563-3576 (2003). cited by applicant .
- Sidhu, S.S., Phage display in pharmaceutical biotechnology, *Curr Opin Biotech* 11:610-616 (2000).

cited by applicant .

Siemering et al., Mutations that suppress the thermosensitivity of green fluorescent protein, *Current Biology* 6:1653-1663 (1996). cited by applicant .

Silva-Cunha et al., W/O/W multiple emulsions of insulin containing a protease inhibitor and an absorption enhancer: biological activity after oral administration to normal and diabetic rats, *Int J Pharm* 169:33-44 (1998). cited by applicant .

Sims et al., Immunopolymerase chain reaction using real-time polymerase chain reaction for detection, *Anal. Biochem.* 281(2):230-2 (2000). cited by applicant .

Slappendel et al., Normal cations and abnormal membrane lipids in the red blood cells of dogs with familial stomatocytosis hypertrophic gastritis, *Blood* 84:904-909 (1994). cited by applicant .

Slob et al., Structural identifiability of PBPK models: practical consequences for modeling strategies and study designs, *Crit Rev Toxicol.* 27(3):261-72 (1997). cited by applicant .

Smith et al., Direct mechanical measurements of the elasticity of single DNA molecules by using magnetic beads, *Science* 258(5085):1122-1126 (1992). cited by applicant .

Smith et al., Fluorescence detection in automated DNA sequence analysis, *Nature* 321 :674-679 (1986). cited by applicant .

Smith et al., Phage display, *Chemical Reviews* 97(2), 391-410 (1997). cited by applicant .

Smith et al., The synthesis of oligonucleotides containing an aliphatic amino group at the 5' terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis, *Nucl. Acid Res.* 13:2399-2412 (1985). cited by applicant .

Smith G.P., Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, *Science* 228(4705): 1315-7(1985). cited by applicant .

Smyth et al., Markers of apoptosis: methods for elucidating the mechanism of apoptotic cell death from the nervous system, *Biotechniques* 32:648-665 (2000). cited by applicant .

Sohn, et al, Capacitance cytometry: Measuring biological cells one by one, *PNAS* 97(20):10687-10690 (2000). cited by applicant .

Somasundaram and Ramalingam, Gain studies of Rhodamine 6G dye doped polymer laser, *J Photochem Photobiol* 125(1-3):93-98 (1999). cited by applicant .

Song et al., A microfluidic system for controlling reaction networks in time, *Angew. Chem. Int. Ed.* 42(7):768-772 (2003). cited by applicant .

Song et al., Experimental Test of Scaling of Mixing by Chaotic Advection in Droplets Moving Through Microfluidic Channels, *App Phy Lett* 83(22):4664-4666 (2003). cited by applicant .

Song, H. and Ismagilov, R.F., Millisecond kinetics on a microfluidic chip using nanoliters of reagents, *J Am Chem Soc.* 125: 14613-14619 (2003). cited by applicant .

Soni and Meller, Progress toward ultrafast DNA sequencing using solid-state nanopores, *Clin Chem* 53:1996-2001 (2007). cited by applicant .

Soumillion et al., Novel concepts for the selection of catalytic activity. *Curr Opin Biotechnol* 12:387-394 (2001). cited by applicant .

Soumillion et al., Selection of B-lactomase on filamentous bacteriophage by catalytic activity, *J Mol Biol*, 237:415-22 (1994). cited by applicant .

Sproat et al., The synthesis of protected 5'-mercapto-2',5'-dideoxyribonucleoside-3'-0-phosphoramidites, uses of 5'-mercapto-oligodeoxyribonucleotides, *Nucleic Acids Res* 15:4837-4848 (1987). cited by applicant .

Stauber, et a., Rapid generation of monoclonal antibody-secreting hybridomas against African horse sickness virus by in vitro immunization and the fusion/cloning technique, *J. Immunol. Meth*

161(2):157-168 (1993). cited by applicant .

Stemmer, W.P., DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. PNAS 91(22):10747-51(1994). cited by applicant .

Stemmer, W.P., Rapid evolution of a protein in vitro by DNA shuffling, Nature 370(6488):389-91 (1994). cited by applicant .

Stober et al., Controlled growth of monodisperse silica spheres in the micron size range, J Colloid and Interface Sci 26(1):62-69 (1968). cited by applicant .

Stofko, H.R. et al., A single step purification for recombinant proteins. Characterization of microtubule associated protein (MAP2) fragment which associates with the type II cAMP-dependent protein kinase, Febs Lett 302: 274-278 (1992). cited by applicant .

Stone et al., Engineering flows in small devices: Microfluidics toward a lab-on-a-chip, Ann. Rev. Fluid Mech. 36:381-441 (2004). cited by applicant .

Strizhkov et al., PCR amplification on a microarray of gel-immobilized oligonucleotides: Detection of bacterial toxin- and drug-resistant genes and their mutations, BioTechniques 29(4):844-857 (2000). cited by applicant .

Stroock et al., Chaotic mixer for microchannels, Science 295(5555):647-651 (2002). cited by applicant .

Studer et al., Fluorous Synthesis: A FluoroPhase Strategy for Improving Separation Efficiency in Organic Synthesis, Science 275: 823-826 (1997). cited by applicant .

Sugiura et al., Effect of Channel Structure on MicroChannel Emulsification, Langmuir 18:5708-5712 (2002). cited by applicant .

Sugiura et al., Interfacial tension driven monodispersed droplet formation from microfabricated channel array Langmuir, 17: 5562-5566 (2001). cited by applicant .

Sundberg et al., Spatially-Addressable Immobilisation of Macromolecules on Solid Supports, J. Am. Chem. Soc, 117:12050-12057 (1995). cited by applicant .

Sung et al. Chip-based microfluidic devices coupled with electrospray ionization-mass spectrometry, Electrophoresis 26:1783-1791 (2005). cited by applicant .

Suzuki et al., Random mutagenesis of thermus aquaticus DNA polymerase I: concordance of immutable sites in vivo with the crystal structure, PNAS USA, 93:96701-9675 (1996). cited by applicant .

Tabatabai and Faghri, A New Two-Phase Flow Map and Transition Boundary Accounting for Surface Tension Effects in Horizontal Miniature and Micro Tubes, J Heat Transfer 123:958-968 (2001). cited by applicant .

Tabatabai et al, Economic feasibility study of polyelectrolyte-enhanced ultrafiltration (PEUF) for water softening, J Membrane Science 100(3):193-207 (1995). cited by applicant .

Tabatabai et al., Reducing Surfactant Adsorption on Carbonate Reservoirs, SPE Reservoir Engineering 8(2):117-122 (1993). cited by applicant .

Tabatabai, Water Softening Using polyelectrolyte-enhanced ultrafiltration, Separation Science Technology 30(2):211-224 (1995). cited by applicant .

Takayama et al., Patterning Cells and Their Environments Using Multiple Laminar Fluid Flows in Capillary Networks, PNAS 96:5545-5548 (1999). cited by applicant .

Takeuchi et al., An Axisymmetric Flow-Focusing Microfluidic Device, Adv. Mater 17(8):1067-1072 (2005). cited by applicant .

Taly et al., Droplets as Microreactors for High-Throughput Biology, ChemBiochem 8(3):263-272 (2007). cited by applicant .

- Thorsen et al., Dynamic pattern formation in a vesicle-generating microfluidic device, *Phys Rev Lett* 86(18):4163-4166 (2001). cited by applicant .
- Thorsen et al., Microfluidic Large-Scale Integration, *Science* 298:580-584 (2002). cited by applicant .
- Tice et al., Effects of viscosity on droplet formation and mixing in microfluidic channels, *Analytica Chimica Acta* 507:73-77 (2004). cited by applicant .
- Tice et al., Formation of droplets and mixing in multiphase microfluidics at low values of the reynolds and the capillary numbers, *Langmuir* 19:9127-9133 (2003). cited by applicant .
- Titomanlio et al., Capillary experiments of flow induced crystallization of HOPE, *AIChE Journal*, 36(1):13-18(1990). cited by applicant .
- Tleugabulova et al., Evaluating formation and growth mechanisms of silica particles using fluorescence anisotropy decay analysis, *Langmuir* 20(14):5924-5932 (2004). cited by applicant .
- Tokatlidis et al., Nascent chains: folding and chaperone intraction during elongation on ribosomes, *Philos Trans R Soc Lond B Biol Sci*, 348:89-95 (1995). cited by applicant .
- Tokeshi et al., ContinuoFlow Chemical Processing on a Microchip by Combining Microunit Operations and a Multiphase Flow NetWO rk, *Anal Chem* 74(7):1565-1571 (2002). cited by applicant .
- Tokumitsu, H. et al., Preparation of gadopentetic acid-loaded chitosan microparticles for gadolinium neutron-capture therapy of cancer by a novel emulsion-droplet coalescence technique, *Chem and Pharm Bull* 47(6):838-842 (1999). cited by applicant .
- Tramontano, A., Catalytic antibodies, *Science* 234(4783):1566-70 (1986). cited by applicant .
- Trindade, T., Nanocrystalline semiconductors: synthesis, properties, and perspectives, *Chem. Mat.* 13:3843-3858 (2001). cited by applicant .
- Tripet, B. et al., Engineering a de novo-designed coiled-coil heterodimerization domain off the rapid detection, purification and characterization of recombinantly expressed peptides and proteins, *Protein Engng.*, 9:1029-42 (1996). cited by applicant .
- Tuerk, C. and Gold, L., Systematic Evolution of Ligands by Exponentid Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase, *Science*, 249:505-10 (1990). cited by applicant .
- Umbanhowar et al., Monodisperse Emulsion Generation via Drop Break Off in a Coflowing Stream, *Langmuir* 16(2):347-351 (2000). cited by applicant .
- Unger et al., Monolithic microfabricated valves and pumps by multilayer soft lithography, *Science* 288(5463):113-116 (2000). cited by applicant .
- Utada, A. et al., Monodisperse double emulsions generated from a microcapillary device, *Science*, 308:537-541 (2005). cited by applicant .
- Vainshtein et al., Peptide rescue of an N-terminal truncation of the stoffel fragment of Taq DNA polymerase, *Protein Science*, 5:1785-92 (1996). cited by applicant .
- Van Bockstaele et al., Prognostic markers in chronic lymphocytic leukemia: a comprehensive review, *Blood Rev* 23(1):25-47 (2009). cited by applicant .
- Van Dilla et al., Cell Microfluorometry: A Method for Rapid Fluorescence Measurement, *Science* 163(3872):1213-1214 (1969). cited by applicant .
- Van Dilla et al., The fluorescent cell photometer: a new method for the rapid measurement of biological cells stained with fluorescent dyes, *Annual Report of the Los Alamos Scientific Laboratory of the University of California (Los Alamos, NM), Biological and Medical Research Group (H-4) of the Health Division, Compiled by D. G. Ott, pp. 100-105, distributed Jan. 23, 1968.* cited by applicant .

- Vanhook et al., Three-dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate, *Biochemistry* 35:6020-6025 (1996). cited by applicant .
- Varga, J.M. et al., Mechanism of allergic cross-reactions-I. Multispecific binding of ligands to a mouse monoclonal anti-DNP IgE antibody. *Mol Immunol* 28(6), 641-54 (1991). cited by applicant .
- Vary, A homogeneous nucleic acid hybridization assay based on strand displacement, *Nucl Acids Res* 15(17):6883-6897 (1987). cited by applicant .
- Venkateswaran et al., Production of Anti-Fibroblast Growth Factor Receptor Monoclonal Antibodies by In Vitro Immunization, *Hybirdoma*, 11(6):729-739 (1992). cited by applicant .
- Venter et al., The sequence of the human genome, *Science* 291(5507):1304-51 (2001). cited by applicant .
- Vogelstein et al., Digital PCR, *PNAS* 96(16):9236-9241 (1999). cited by applicant .
- Voss, E.W., Kinetic measurements of molecular interactions by spectrofluorometry, *J Mol Recognit*, 6:51-58 (1993). cited by applicant .
- Wahler, D. et al., Novel methods for biocatalyst screening. *Curr Opin Chem Biol*, 5: 152-158 (2001). cited by applicant .
- Walde, P. et al., Oparin's reactions revisited: enzymatic synthesis of poly(adenylic acid) in micelles and self-reproducing vesicles. *J Am Chem Soc*, 116: 7541-7547 (1994). cited by applicant .
- Walde, P. et al., Spectroscopic and kinetic studies of lipases solubilized in reverse micelles, *Biochemistry*, 32(15):4029-34 (1993). cited by applicant .
- Walde, P. et al., Structure and activity of trypsin in reverse micelles, *Eur J Biochem*, 173(2):401-9 (1988). cited by applicant .
- Walker et al., Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system, *PNAS* 89(1):392-6 (1992). cited by applicant .
- Walker et al., Strand displacement amplification--an isothermal, in vitro DNA amplification technique, *Nucleic Acid Res*, 20(7):1691-6 (1992). cited by applicant .
- Wang et al., DEP actuated nanoliter droplet dispensing using feedback control, *Lab on a Chip* 9:901-909 (2008). cited by applicant .
- Wang et al., Preparation of Titania Particles Utilizing the Insoluble Phase Interface in a MicroChannel Reactor, *Chemical Communications* 14:1462-1463 (2002). cited by applicant .
- Wang, A.M. et al., Quantitation of mRNA by the polymerase chain reaction. *Proc natl Aced Sci USA* 86(24), 9717-21 (1989). cited by applicant .
- Wang, G.T. et al., Design and synthesis of new fluorogenic HIV protease substrates based on resonance energy transfer, *Tetrahedron Lett.*, 31:6493 (1990). cited by applicant .
- Warburton, B., Microcapsules for Multiple Emulsions, Encapsulation and Controlled Release, *Spec Publ R Soc Chem*, 35-51 (1993). cited by applicant .
- Wasserman et al., Structure and reactivity of allyl-siloxane monolayers formed by reaction of allyltrichlorosilanes on silicon substrates, *Langmuir* 5:1074-1087 (1989). cited by applicant .
- Weil. et al., Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA, *Cell*, 18(2):469-84 (1979). cited by applicant .
- Werle et al., Convenient single-step, one tube purification of PCR products for direct sequencing, *Nucl Acids Res* 22(20):4354-4355 (1994). cited by applicant .
- Wetmur et al., Molecular haplotyping by linking emulsion PCR: analysis of paraoxonase 1

haplotypes and phenotypes, *Nucleic Acids Res* 33(8):2615-2619 (2005). cited by applicant .

Wick et al., Enzyme-containing liposomes can endogenously produce membrane-constituting lipids, *Chem Biol* 3(4):277-85 (1996). cited by applicant .

Widersten and Mannervik, Glutathione Transferases with Novel Active Sites Isolated by Phage Display from a Library of Random Mutants, *J Mol Biol* 250(2):115-22 (1995). cited by applicant .

Wiggins et al., Foundations of chaotic mixing, *Philos Transact A Math Phys Eng Sci* 362(1818):937-70 (2004). cited by applicant .

Williams et al., Amplification of complex gene libraries by emulsion PCR, *Nature Methods* 3(7):545-550 (2006). cited by applicant .

Williams et al., Methotrexate, a high-affinity pseudosubstrate of dihydrofolate reductase, *Biochemistry*, 18(12):2567-73 (1979). cited by applicant .

Wilson, D.S. and Szostak, J.W., In vitro selection of functional nucleic acids, *Ann. Rev. Biochem* 68: 611-647 (1999). cited by applicant .

Winter et al., Making antibodies by phage display technology, *Annu Rev Immunol* 12:433-55 (1994). cited by applicant .

Wittrup, K.D., Protein engineering by cell-surface display. *Curr Opin Biotechnology*, 12: 395-399 (2001). cited by applicant .

Wolff et al., Integrating advanced functionality in a microfabricated high-throughput fluorescent-activated cell sorter, *Lab Chip*, 3(1): 22-27 (2003). cited by applicant .

Wronski et al., Two-color, fluorescence-based microplate assay for apoptosis detection. *Biotechniques*, 32:666-668 (2002). cited by applicant .

Wu et al., The ligation amplification reaction (LAR)--amplification of specific DNA sequences using sequential rounds of template-dependent ligation, *Genomics* 4(4):560-9 (1989). cited by applicant .

Wyatt et al., Synthesis and purification of large amounts of RNA oligonucleotides, *Biotechniques* 11(6):764-9 (1991). cited by applicant .

Xia and Whitesides, Soft Lithography, *Angew. Chem. Int. Ed.* 37:550-575 (1998). cited by applicant .

Xia and Whitesides, Soft Lithography, *Ann. Rev. Mat. Sci.* 28:153-184 (1998). cited by applicant .

Xu, S. et al., Generation of monodisperse particles by using microfluidics: control over size, shape, and composition, *Angew. Chem. Int. Ed.* 44:724-728 (2005). cited by applicant .

Yamagishi, J. et al., Mutational analysis of structure-activity relationships in human tumor necrosis factor- α , *Protein Eng.* 3:713-9 (1990). cited by applicant .

Yamaguchi et al., Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives, *Journal of Controlled Release*, 81(3): 235-249 (2002). cited by applicant .

Yelamos, J. et al., Targeting of non-Ig sequences in place of the V segment by somatic hypermutation. *Nature* 376(6537):225-9 (1995). cited by applicant .

Yershov et al., DNA analysis and diagnostics on oligonucleotide microchips, *PNAS* 93(10):4913-4918 (1996). cited by applicant .

Yonezawa et al., DNA display for in vitro selection of diverse peptide libraries, *Nucleic Acids Research*, 31(19): e118 (2003). cited by applicant .

Yu et al. Responsive biomimetic hydrogel valve for microfluidics. *Appl. Phys. Lett* 78:2589-2591 (2001). cited by applicant .

Yu et al., Quantum dot and silica nanoparticle doped polymer optical fibers, *Optics Express* 15(16):9989-9994 (2007). cited by applicant .

Yu et al., Specific inhibition of PCR by non-extendable oligonucleotides using a 5' to 3' exonuclease-

Parent Case Text

REFERENCE TO RELATED APPLICATIONS

This non-provisional application claims priority to U.S. Provisional Application No. 61/388,937, filed Oct. 1, 2010, U.S. Provisional Application No. 61/347,158, filed May 21, 2010, U.S. Provisional Application No. 61/331,490, filed, May 5, 2010, and U.S. Provisional Application No. 61/304,163, filed Feb. 12, 2010, the contents of which are each incorporated by reference herein in their entireties.

Claims

What is claimed is:

1. A method for analyzing a target nucleic acid, the method comprising the steps of: forming droplets containing a single target nucleic acid and one or more amplification reagents; amplifying the target in the droplets; distinguishing droplets containing amplicon from the target and amplicon from a variant of the target generated by polymerase error by using two differently labeled-hybridization probes, one hybridizing to the target and one hybridizing to a specific variant of the target; and analyzing target amplicons.
2. The method according to claim 1, wherein said amplifying step is a polymerase chain reaction and the one or more amplification reagents includes one or more primer pairs.
3. The method according to claim 1, wherein said distinguishing step comprises flowing said droplets in a microfluidic channel.
4. The method according to claim 1, wherein said analyzing step comprises detecting said amplicons by hybridization to detectably-labeled probes.
5. The method according to claim 1, wherein said analyzing step is conducted on amplicon from droplets that were not distinguished in said distinguishing step.
6. The method according to claim 1, wherein said forming step comprises: flowing a stream of first sample fluid comprising nucleic acids such that it intersects two opposing streams of flowing carrier fluid, wherein said carrier fluid is immiscible with said sample fluid, thereby forming a plurality of first droplets comprising the first sample fluid; and merging each of the plurality of first droplets comprising the first sample fluid with a portion of a second fluid comprising one or more amplification reagents, wherein the portion of a second fluid optionally is a droplet.
7. The method according to claim 6, wherein said carrier fluid is oil.
8. The method according to claim 7, wherein said oil comprises a surfactant.
9. The method according to claim 8, wherein the surfactant is a fluorosurfactant.

10. The method according to claim 1, wherein said analyzing step comprises: determining a number of droplets that contain only wild-type target; determining a number of droplets that contain only a variant of the target.
11. The method according to claim 10, wherein presence of droplets containing only said variant is indicative of a disease.
12. The method according to claim 11, wherein the disease is cancer.
13. The method according to claim 10, wherein the variant is an allelic variant.
14. The method according to claim 13, wherein the allelic variant is a single nucleotide polymorphism.

Description

FIELD OF THE INVENTION

The invention generally relates to droplet based digital PCR and methods for analyzing a target nucleic acid using the same.

BACKGROUND

Assays have been developed that rely on analyzing nucleic acid molecules from bodily fluids for the presence of mutations, thus leading to early diagnosis of certain diseases such as cancer. In a typical bodily fluid sample however, any abnormal nucleic acids containing mutations of interest are often present in small amounts (e.g., less than 1%) relative to a total amount of nucleic acid in the bodily fluid sample. This can result in a failure to detect the small amount of abnormal nucleic acid due to stochastic sampling bias.

The advent of PCR and real-time PCR methodologies has greatly improved the analysis of nucleic acids from both throughput and quantitative perspectives. While traditional PCR techniques typically rely on end-point, and sometimes semi-quantitative, analysis of amplified DNA targets via agarose gel electrophoresis, real-time PCR (or qPCR) methods are geared toward accurately quantifying exponential amplification as the reaction progresses. qPCR reactions are monitored either using a variety of highly sequence specific fluorescent probe technologies, or by using non-specific DNA intercalating fluorogenic dyes.

As the need for higher throughput in analyzing multiple targets in parallel continues to escalate in the fields of genomics and genetics, and as the need for more efficient use of sample grows in medically related fields such as diagnostics, the ability to perform and quantify multiple amplifications simultaneously within the same reaction volume (multiplexing) is paramount for both PCR and qPCR. While end-point PCR can support a high level of amplicon multiplexing, such ample capacity for multiplexing probe-based qPCR reactions remains elusive for a number of reasons. For example, most commercial real-time thermal cyclers only support up to four differently colored fluorophores for detection as a consequence of the limited spectral resolution of common fluorophores, translating into a multiplexing capacity of 4.times.. Additionally, while optimization of single target primer/probe reactions is now standard practice, combining primers and probes for multiple reactions changes the

thermodynamic efficiencies and/or chemical kinetics, necessitating potentially extensive troubleshooting and optimization. Very high multiplexing of greater than 100.times. has been demonstrated in a "one of many" detection format for pathogen identification using "sloppy" molecular beacons and melting points as fingerprints, however the approach is restricted to applications with a slim likelihood of the presence of multiple simultaneous targets. A half-multiplexing method achieved 19.times. in a two step reaction with general multiplexed preamplification in the first step, followed by separate single-plex quantitative PCR in the second step. However a general purpose single-pot solution to qPCR multiplexing does not yet exist.

Digital PCR (dPCR) is an alternative quantitation method in which dilute samples are divided into many separate reactions. See for example, Brown et al. (U.S. Pat. Nos. 6,143,496 and 6,391,559) and Vogelstein et al. (U.S. Pat. Nos. 6,440,706, 6,753,147, and 7,824,889), the content of each of which is incorporated by reference herein in its entirety. The distribution from background of target DNA molecules among the reactions follows Poisson statistics, and at so called "terminal dilution" the vast majority of reactions contain either one or zero target DNA molecules for practical intents and purposes. In another case, at so called "limiting dilution" some reactions contain zero DNA molecules, some reactions contain one molecule, and frequently some other reactions contain multiple molecules, following the Poisson distribution. It is understood that terminal dilution and limiting dilution are useful concepts for describing DNA loading in reaction vessels, but they have no formal mathematical definition, nor are they necessarily mutually exclusive. Ideally, at terminal dilution, the number of PCR positive reactions (PCR(+)) equals the number of template molecules originally present. At limiting dilution, Poisson statistics are used to uncover the underlying amount of DNA. The principle advantage of digital compared to qPCR is that it avoids any need to interpret the time dependence of fluorescence intensity--an analog signal--along with the main underlying uncertainty of non-exponential amplification during early cycles.

SUMMARY

The invention generally relates to the manipulation of nucleic acid in droplets, and in particular, nucleic acid-amplification and detection. In one aspect, the invention provides a droplet that contains a single nucleic acid template and a plurality of primer pairs specific for multiple target sites on the template. The single nucleic acid template can be DNA (e.g., genomic DNA, cDNA, etc.) or RNA. The template is amplified in the droplet for detection; and may preferably be amplified using a plurality of primer pairs as described herein.

The ability to amplify and detect single nucleic acids in droplets enables digital PCR, detection, counting, and differentiation among nucleic acids, especially those present in heterogeneous samples. Thus, the invention applies to digital amplification techniques and, in specific embodiments enables multiplex PCR in droplets. For example, multiplexing primers in droplets enables the simultaneous increase in the number of PCR droplets while keeping the amount of input DNA the same or lower and generate the same or greater amplicon yield. This results in an overall increase in the amount of PCR positive droplets and amplicon yield without the consumption of more DNA. Even though the number of PCR primer pairs per droplet is greater than one, there is only one template molecule per droplet, and thus, in some implementations, there is only one primer pair per droplet that is being utilized at one time. As such, the advantages of droplet PCR for eliminating bias from either allele specific PCR or competition between different amplicons is maintained. However, as described below in relation to detection of haplotypes, other implementations advantageously allow detection of multiple loci on a single template using multiple primer pairs, preferably designed to minimize bias.

Microfluidic droplets for multiplex analysis according to the invention contain a plurality of probes that hybridize

to amplicons produced in the droplets. Preferably, the droplet contains two or more probes, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 60, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 500, or more probes. Certain members of the plurality of probes include a detectable label. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The detectable label is preferably a fluorescent label. The plurality of probes can include one or more groups of probes at varying concentrations. The one or more groups of probes can include the same detectable label which will vary in intensity upon detection, due to the varying probe concentrations. The droplets of the invention can further contain one or more reagents for conducting a polymerase chain reaction, such as a DNA or RNA polymerase, and/or dNTPs.

The present invention additionally relates to a method for detecting a plurality of targets in a biological sample using digital PCR in microfluidic droplets. The sample may be a human tissue or body fluid. Exemplary body fluids pus, sputum, semen, urine, blood, saliva, and cerebrospinal fluid.

One or more droplets are formed, each containing a single nucleic acid template and a heterogeneous mixture of primer pairs and probes, each specific for multiple target sites on the template. For example, a first fluid (either continuous, or discontinuous as in droplets) containing a single nucleic acid template (DNA or RNA) is merged with a second fluid (also either continuous, or discontinuous as in droplets) containing a plurality of primer pairs and a plurality of probes, each specific for multiple targets sites on the nucleic acid template to form a droplet containing the single nucleic acid template and a heterogeneous mixture of primer pairs and probes. The second fluid can also contain reagents for conducting a PCR reaction, such as a polymerase and dNTPs.

Certain members of the plurality of probes include a detectable label. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The detectable label is preferably a fluorescent label. The plurality of probes can include one or more groups of probes at varying concentrations. The one or more groups of probes can include the same detectable label which varies in intensity upon detection, due to the varying probe concentrations.

The first and second fluids can each be in droplet form. Any technique known in the art for forming droplets may be used with methods of the invention. An exemplary method involves flowing a stream of the sample fluid containing the nucleic acid template such that it intersects two opposing streams of flowing carrier fluid. The carrier fluid is immiscible with the sample fluid. Intersection of the sample fluid with the two opposing streams of flowing carrier fluid results in partitioning of the sample fluid into individual sample droplets containing the first fluid. The carrier fluid may be any fluid that is immiscible with the sample fluid. An exemplary carrier fluid is oil. In certain embodiments, the carrier fluid includes a surfactant, such as a fluorosurfactant. The same method may be applied to create individual droplets from the second fluid containing the primer pairs (and, in some implementations, the amplification reagents). Either the droplets containing the first fluid, the droplets containing the second fluid, or both, may be formed and then stored in a library for later merging, aspects of certain implementations of which are described in U.S. patent application Ser. No. 12/504,764, hereby incorporated herein in its entirety for all purposes. Once formed, droplets containing the first and second fluids can be merged to form single droplets containing the single nucleic acid template and heterogeneous mixture of primer pairs and probes. Merging can be accomplished, for example, in the presence of an electric field. Moreover, it is not required that both fluids be in the form of droplets when merging takes place. One exemplary method for merging of fluid portions with droplets is taught, for example, in co-pending U.S. Patent Application No. 61/441,985, filed on even date herewith.

The nucleic acid template in each of the merged/formed droplets is amplified, e.g., by thermocycling the droplets under temperatures/conditions sufficient to conduct a PCR reaction. The resulting amplicons in the droplets can then be analyzed. For example, the presence or absence of the plurality of targets in the one or more droplets is detected optically, e.g., by the detectable label on the plurality of probes.

The invention further relates to methods for analyzing a target nucleic acid. More particularly, methods of the invention are able to detect polymerase errors that occur during a PCR reaction and are able to exclude from analysis amplification products that are a result of a polymerase error. Methods of the invention are particularly useful in digital PCR where a polymerase error may result in a partitioned section of sample being incorrectly identified as containing a mutant allele, i.e., a false positive. Such false positives greatly impact the validity and precision of digital PCR results. Methods of the invention are able to uniquely detect multiple targets with the same optical color. Methods of the invention are particularly useful in digital PCR where it is desirable to identify multiple different target molecules that may be present in the starting test fluid.

Methods of the invention involve forming sample droplets containing target nucleic acid. Ideally, methods of the invention comprise forming droplets for digital PCR. Preferred digital PCR droplets contain one copy of a nucleic acid to be amplified, although they may contain multiple copies of the same nucleic acid sequence. Any technique known in the art for forming sample droplets may be used with methods of the invention. One exemplary method involves flowing a stream of sample fluid including nucleic acids such that it intersects two opposing streams of flowing carrier fluid. The carrier fluid is immiscible with the sample fluid. Intersection of the sample fluid with the two opposing streams of flowing carrier fluid results in partitioning of the sample fluid into individual sample droplets. The carrier fluid may be any fluid that is immiscible with the sample fluid. An exemplary carrier fluid is oil. In certain embodiments, the carrier fluid includes a surfactant, such as a fluorosurfactant.

The targets are then amplified in the droplets. Any method known in the art may be used to amplify the target nucleic acids either linearly or exponentially. A preferred method is the polymerase chain reaction (PCR). For purposes of the invention, any amplification technique commonly known in the art may be implemented such as rolling circle amplification, isothermal amplification, or any combination of amplification methods using loci specific primers, nested-primers, or random primers (such primers, and/or primers used for PCR, are included in the term "amplification reagents"). Once amplified, droplets containing amplicon from the target and amplicon from a variant of the target are excluded. One method to exclude droplets that contain a heterogeneous population of amplicons from droplets that contain a homogeneous population of amplicons includes hybridizing detectably-labeled probes to the amplicons, flowing the droplets through a microfluidic channel, and excluding those droplets in which both amplicon from the target and amplicon from a variant of the target are detected.

Once droplets containing a heterogeneous population of amplicons are excluded, droplets that contain a homogeneous population of amplicons are analyzed. Any analytical technique known in the art may be used. In certain embodiments, analyzing the droplets involves determining a number of droplets that contain only wild-type target, and determining a number of droplets that contain only a variant of the target. Generally, the presence of droplets containing only the variant is indicative of a disease, such as cancer. The variant may be an allelic variant. An exemplary allelic variant is a single nucleotide polymorphism. The variant may also be a specific haplotype. Haplotypes refer to the presence of two or more variants on the same nucleic acid strand. Haplotypes can be more informative or predictive than genotypes when used to determine such things as the presence or

severity of disease, response to drug therapy or drug resistance of bacterial or viral infections. Because each droplet contains only one template strand it is an ideal vessel for the determination of haplotypes. The detection of two or more variants in a single droplet that contains a single intact nucleic acid strand identifies the haplotype of the variants on that strand. The presence of two or more markers in the same droplet can be identified by such methods as the presence of dyes of multiple colors or the increase in the intensity of a single dye or a combination of both. Any method that allows the identification of multiple variants in a single droplet enables the determination of a sample's haplotype.

In accordance with some implementations of the invention, a method is provided for analyzing a target nucleic acid that includes compartmentalizing a first fluid into portions, each portion containing a single target nucleic acid; amplifying the target in the portions; excluding portions containing amplicon from the target and amplicon from a variant of the target; and analyzing target amplicons.

In other aspects, the invention generally provides methods for detecting a recurrence of a cancer in a patient. Those methods may involve forming sample droplets containing a single target nucleic acid derived from a patient sample, flowing the sample droplets through a channel, amplifying the target in the droplets, detecting amplified target in the droplets, excluding droplets including a heterogeneous population of amplicons, and analyzing non-excluded droplets to determine the presence of mutant alleles indicative of recurrence. In certain embodiments, the analyzing step includes capturing amplicon obtained from the droplets using labeled capture probes. The sample may be a human tissue or body fluid. Exemplary body fluids are pus, sputum, semen, urine, blood, saliva, stool, and cerebrospinal fluid. In other aspects of the invention generally provide a method for forensic identification of low levels of target nucleic acid in an environment having multiple other sources of nucleic acid. Such methods may also be practiced using fluids compartmentalized in containers other than or in addition to droplets.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts a droplet formation device.

FIG. 2 depicts a portion of the droplet formation device of FIG. 1.

FIG. 3 depicts an exemplary microfluidic system for droplet generation and readout. FIG. 3a depicts the droplet generation chip; FIG. 3b depicts the droplet spacing for readout; and FIG. 3c depicts a cartoon of droplet readout by fluorescence.

FIG. 4 depicts the serial dilution of template DNA quantified by dPCR. FIG. 4a shows droplet fluorescence during readout for the most concentrated sample. Each discrete burst of fluorescence corresponded to an individual droplet. Two different groups of droplets were evident: PCR(+) droplets peaking at .about.0.8 V and PCR(-) droplets at .about.0.1 V; FIG. 4b shows a histogram of the peak fluorescence intensities of droplets from the complete data trace in (a). PCR(+) and PCR(-) droplets appeared as two very distinct populations centered at 0.78 and 0.10 V, respectively; FIG. 4c shows the serial dilution of template DNA. Open circles: measured occupancies; solid line: the best fit to Eqn 2 ($A=0.15$, $f=4.8$, $R_{\text{sup.2}}=0.9999$).

FIG. 5A is a schematic representation of a droplet having 5 sets of primers for PCR amplification of a template sequence and 5 probes, each labeled with a fluorescent dye, that binds specifically to the amplified sequences;

FIG. 5B is a time trace of fluorescence intensity detected from droplets after PCR amplification; FIG. 5C is a scatter plot showing clusters representing droplets that contain specific amplified sequences (TERT, RNaseP, E1a, SMN1 and SMN2).

FIG. 6A is a schematic representation of a droplet having 5 sets of primers for PCR amplification of a template sequence and 5 probes, each labeled with a fluorescent dye, that binds specifically to the amplified sequences; FIG. 6B is a scatter plot showing clusters representing droplets that contain specific amplified sequences (TERT, 815A, RNaseP, E1a, and 815G); FIG. 6C is a table showing the copy number of specific sequences shown in FIG. 6B.

FIG. 7 is a schematic depicting one-color detection of a genetic sequence with a microfluidic device.

FIG. 8 is a schematic depicting two-color detection of two genetic sequences with a microfluidic device.

FIG. 9 is a schematic depicting two-color detection of three genetic sequences with a microfluidic device.

FIG. 10 shows two dot plots depicting clusters of genetic sequences detected through fluorescence intensity. Left panel is a dot plot showing four clusters. Block for SMN1 sequence was present. Top left: microdroplets containing the reference sequence (SMARCC1); bottom left: microdroplets not containing any sequence; bottom middle: microdroplets containing sequence for SMN1; and bottom right: microdroplets containing sequence for SMN2. Right panel is a dot plot showing four clusters. No block for SMN1 sequence was present. Top left: microdroplets containing the reference sequence (SMARCC1); bottom left: microdroplets not containing any sequence; bottom middle: microdroplets containing sequence for SMN1; and bottom right: microdroplets containing sequence for SMN2. The shift of the bottom middle cluster in right panel as compared to left panel confirms that fluorescence intensity provides a very sensitive measurement for the presence of a sequence.

FIG. 11 depicts histograms of a duplex gene copy number assay using only one type of fluorophore by digital PCR; FIG. 11a depicts a histogram of droplet peak fluorescence intensities; FIG. 11b shows a comparison of gene copy numbers measured by monochromatic dPCR.

FIG. 12 is a schematic for tuning the intensity of a detectable label to a particular target with a microfluidic device.

FIG. 13 is a line graph depicting the linear dependence of droplet fluorescence intensity on probe concentration (Line, best linear fit ($y = -0.092x + 0.082$, $R_{\text{sup.2}} = 0.995$)).

FIG. 14 depicts a 5-plex dPCR assay for spinal muscular atrophy with only two fluorophores. FIG. 14a is a 2D histogram of droplet fluorescence intensities, shown as a heat map, for the 5-plex assay against the synthetic model chromosome for validation. The six well resolved droplet populations corresponded to the five individual assays plus the empty droplets; FIG. 14b shows the results of the SMA pilot study.

FIG. 15 depicts a 9-plex dPCR assay for spinal muscular atrophy with only two fluorophores, showing the process of optimizing droplet intensities. FIGS. 15a and 15b show 2-D histograms of droplet fluorescence intensity, shown as heat maps with hotter colors representing higher droplet counts, for the 9-plex assay against the synthetic model chromosome ((a)=Before optimization; (b)=after optimization).

FIG. 16 depicts an optical schematic for combining optical labels with multiplexing.

FIG. 17 depicts a dPCR assay combining multiplexing with optical labels using co-flow microfluidics. The contributions from all droplets are shown, that is, from three different triplex assays. (Both panels) 2-D histograms shown as heat maps with hotter colors representing higher droplet counts. (Left panel) histogram of optical labels, i.e. fluorescence intensities of droplets measured at wavelengths for the two fluorophores comprising the optical labels. (Right panel) assay histogram, i.e. fluorescence intensities of droplets measured at wavelengths suitable for FAM detection (x-axis), and VIC detection (y-axis). Both histograms were compensated for spectral overlap by standard techniques.

FIG. 18 shows single assay selections using optical labels. Selections were taken from all of the droplets from FIG. 17. Each of the three different selections in panels A-C were for optical labels encoding the same assay (TERT, SMN1, and SMN2). Histograms are as described in FIG. 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

FIG. 19 shows single assay selections using optical labels. Selections were taken from all of the droplets from FIG. 17. Each of the three different selections in panels A-C was for optical labels encoding the same assay (TERT, c.5C from SMN1, and BCKDHA). Histograms are as described in FIG. 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

FIG. 20 shows single assay selections using optical labels. Selections were taken from all of the droplets from FIG. 17. Each of the three different selections in panels A-C was for optical labels encoding the same assay (TERT, c.88G from SMN1, and RNaseP). Histograms are as described in FIG. 17. (Left histograms, optical labels) Superimposed lines demark the bounding box for selecting a single optical label. (Right histograms, assay) Only droplets containing the selected optical label are displayed.

FIG. 21 depicts a dPCR assay combining multiplexing with optical labels using droplet merging.

FIG. 22 is a schematic showing haplotype detection in droplets.

DETAILED DESCRIPTION

The invention provides materials and methods for analysis of biomolecules. In one aspect, the invention provides for digital analysis in droplets, such as microfluidic droplets. The invention allows digital PCR to be conducted and provides for significantly reduced or eliminated errors.

Ideally, the sensitivity of digital PCR is limited only by the number of independent amplifications that can be analyzed, which has motivated the development of several ultra-high throughput miniaturized methods allowing millions of single molecule PCR reactions to be performed in parallel (discussed in detail elsewhere). In a preferred embodiment of the invention, digital PCR is performed in aqueous droplets separated by oil using a microfluidics system. In another preferred embodiment, the oil is a fluorinated oil such as the Fluorinert oils (3M). In a still more preferred embodiment the fluorinated oil contains a surfactant, such as PFPE-PEG-PFPE triblock

Methods of the invention involve novel strategies for performing multiple different amplification reactions on the same sample simultaneously to quantify the abundance of multiple different DNA targets, commonly known to those familiar with the art as "multiplexing". Methods of the invention for multiplexing dPCR assays promise greater plexity--the number of simultaneous reactions--than possible with existing qPCR or dPCR techniques. It is based on the singular nature of amplifications at terminal or limiting dilution that arises because most often only a single target allele is ever present in any one droplet even when multiple primers/probes targeting different alleles are present. This alleviates the complications that otherwise plague simultaneous competing reactions, such as varying arrival time into the exponential stage and unintended interactions between primers.

Reactions within microfluidic droplets yield very uniform fluorescence intensity at the end point, and ultimately the intensity depends on the efficiency of probe hydrolysis. Thus, in another aspect of the methods of the invention, different reactions with different efficiencies can be discriminated on the basis of end point fluorescence intensity alone even if they have the same color. Furthermore, in another method of the invention, the efficiencies can be tuned simply by adjusting the probe concentration, resulting in an easy-to-use and general purpose method for multiplexing. In one demonstration of the invention, a 5-plex TaqMan.RTM. dPCR assay worked "right out of the box", in contrast to lengthy optimizations that typify qPCR multiplexing to this degree. In another aspect of the invention, adding multiple colors increases the number of possible reactions geometrically, rather than linearly as with qPCR, because individual reactions can be labeled with multiple fluorophores. As an example, two fluorophores (VIC and FAM) were used to distinguish five different reactions in one implementation of the invention.

Methods of the invention involve forming sample droplets containing a single target nucleic acid, amplifying the target in the droplets, excluding droplets containing amplicon from the target and amplicon from a variant of the target, and analyzing target amplicons.

Nucleic Acid Target Molecules

Nucleic acid molecules include deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA). Nucleic acid molecules can be synthetic or derived from naturally occurring sources. In one embodiment, nucleic acid molecules are isolated from a biological sample containing a variety of other components, such as proteins, lipids and non-template nucleic acids. Nucleic acid template molecules can be obtained from any cellular material, obtained from an animal, plant, bacterium, fungus, or any other cellular organism. In certain embodiments, the nucleic acid molecules are obtained from a single cell. Biological samples for use in the present invention include viral particles or preparations. Nucleic acid molecules can be obtained directly from an organism or from a biological sample obtained from an organism, e.g., from blood, urine, cerebrospinal fluid, seminal fluid, saliva, sputum, stool and tissue. Any tissue or body fluid specimen may be used as a source for nucleic acid for use in the invention. Nucleic acid molecules can also be isolated from cultured cells, such as a primary cell culture or a cell line. The cells or tissues from which template nucleic acids are obtained can be infected with a virus or other intracellular pathogen. A sample can also be total RNA extracted from a biological specimen, a cDNA library, viral, or genomic DNA. In certain embodiments, the nucleic acid molecules are bound as to other target molecules such as proteins, enzymes, substrates, antibodies, binding agents, beads, small molecules, peptides, or any other molecule and serve as a surrogate for quantifying and/or detecting the target molecule.

Generally, nucleic acid can be extracted from a biological sample by a variety of techniques such as those described by Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., pp. 280-281 (1982). Nucleic acid molecules may be single-stranded, double-stranded, or double-stranded with single-stranded regions (for example, stem- and loop-structures).

Droplet Formation

Methods of the invention involve forming sample droplets where some droplets contain zero target nucleic acid molecules, some droplets contain one target nucleic acid molecule, and some droplets may or may not contain multiple nucleic acid molecules (corresponding to limiting or terminal dilution, respectively, as defined above). In the preferred embodiment, the distribution of molecules within droplets obeys the Poisson distribution. However, methods for non-Poisson loading of droplets are known to those familiar with the art, and include but are not limited to active sorting of droplets, such as by laser-induced fluorescence, or by passive one-to-one loading. The description that follows assumes Poisson loading of droplets, but such description is not intended to exclude non-Poisson loading, as the invention is compatible with all distributions of DNA loading that conform to limiting or terminal dilution.

The droplets are aqueous droplets that are surrounded by an immiscible carrier fluid. Methods of forming such droplets are shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Stone et al. (U.S. Pat. No. 7,708,949 and U.S. patent application number 2010/0172803), Anderson et al. (U.S. Pat. No. 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc. The content of each of which is incorporated by reference herein in its entirety.

FIG. 1 shows an exemplary embodiment of a device 100 for droplet formation. Device 100 includes an inlet channel 101, and outlet channel 102, and two carrier fluid channels 103 and 104. Channels 101, 102, 103, and 104 meet at a junction 105. Inlet channel 101 flows sample fluid to the junction 105. Carrier fluid channels 103

and 104 flow a carrier fluid that is immiscible with the sample fluid to the junction 105. Inlet channel 101 narrows at its distal portion wherein it connects to junction 105 (See FIG. 2). Inlet channel 101 is oriented to be perpendicular to carrier fluid channels 103 and 104. Droplets are formed as sample fluid flows from inlet channel 101 to junction 105, where the sample fluid interacts with flowing carrier fluid provided to the junction 105 by carrier fluid channels 103 and 104. Outlet channel 102 receives the droplets of sample fluid surrounded by carrier fluid.

The sample fluid is typically an aqueous buffer solution, such as ultrapure water (e.g., 18 mega-ohm resistivity, obtained, for example by column chromatography), 10 mM Tris HCl and 1 mM EDTA (TE) buffer, phosphate buffer saline (PBS) or acetate buffer. Any liquid or buffer that is physiologically compatible with nucleic acid molecules can be used. The carrier fluid is one that is immiscible with the sample fluid. The carrier fluid can be a non-polar solvent, decane (e.g., tetradecane or hexadecane), fluorocarbon oil, silicone oil or another oil (for example, mineral oil).

In certain embodiments, the carrier fluid contains one or more additives, such as agents which increase, reduce, or otherwise create non-Newtonian surface tensions (surfactants) and/or stabilize droplets against spontaneous coalescence on contact. Surfactants can include Tween, Span, fluorosurfactants, and other agents that are soluble in oil relative to water. In some applications, performance is improved by adding a second surfactant, or other agent, such as a polymer or other additive, to the sample fluid. Surfactants can aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel. This can affect droplet volume and periodicity, or the rate or frequency at which droplets break off into an intersecting channel. Furthermore, the surfactant can serve to stabilize aqueous emulsions in fluorinated oils from coalescing.

In certain embodiments, the droplets may be coated with a surfactant or a mixture of surfactants. Preferred surfactants that may be added to the carrier fluid include, but are not limited to, surfactants such as sorbitan-based carboxylic acid esters (e.g., the "Span" surfactants, Fluka Chemika), including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and sorbitan monooleate (Span 80), and perfluorinated polyethers (e.g., DuPont Krytox 157 FSL, FSM, and/or FSH). Other non-limiting examples of non-ionic surfactants which may be used include polyoxyethylenated alkylphenols (for example, nonyl-, p-dodecyl-, and dinonylphenols), polyoxyethylenated straight chain alcohols, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long chain carboxylic acid esters (for example, glyceryl and polyglyceryl esters of natural fatty acids, propylene glycol, sorbitol, polyoxyethylenated sorbitol esters, polyoxyethylene glycol esters, etc.) and alkanolamines (e.g., diethanolamine-fatty acid condensates and isopropanolamine-fatty acid condensates).

In certain embodiments, the carrier fluid may be caused to flow through the outlet channel so that the surfactant in the carrier fluid coats the channel walls. In one embodiment, the fluorosurfactant can be prepared by reacting the perfluorinated polyether DuPont Krytox 157 FSL, FSM, or FSH with aqueous ammonium hydroxide in a volatile fluorinated solvent. The solvent and residual water and ammonia can be removed with a rotary evaporator. The surfactant can then be dissolved (e.g., 2.5 wt %) in a fluorinated oil (e.g., Fluorinert (3M)), which then serves as the carrier fluid.

One approach to merging sample fluids, using a device called a lambda injector, involves forming a droplet, and contacting the droplet with a fluid stream, in which a portion of the fluid stream integrates with the droplet to form

a mixed droplet. In this approach, only one phase needs to reach a merge area in a form of a droplet. Further description of such method is shown in the co-owned and co-pending U.S. patent application to Yurkovetsky, et al. (U.S. patent application Ser. No. 61/441,985), the content of which is incorporated by reference herein in its entirety.

According to a method for operating the lambda injector, a droplet is formed as described above. After formation of the sample droplet from the first sample fluid, the droplet is contacted with a flow of a second sample fluid stream. Contact between the droplet and the fluid stream results in a portion of the fluid stream integrating with the droplet to form a mixed droplet.

The droplets of the first sample fluid flow through a first channel separated from each other by immiscible carrier fluid and suspended in the immiscible carrier fluid. The droplets are delivered to the merge area, i.e., junction of the first channel with the second channel, by a pressure-driven flow generated by a positive displacement pump. While droplet arrives at the merge area, a bolus of a second sample fluid is protruding from an opening of the second channel into the first channel. Preferably, the channels are oriented perpendicular to each other. However, any angle that results in an intersection of the channels may be used.

The bolus of the second sample fluid stream continues to increase in size due to pumping action of a positive displacement pump connected to channel, which outputs a steady stream of the second sample fluid into the merge area. The flowing droplet containing the first sample fluid eventually contacts the bolus of the second sample fluid that is protruding into the first channel. Contact between the two sample fluids results in a portion of the second sample fluid being segmented from the second sample fluid stream and joining with the first sample fluid droplet to form a mixed droplet. In certain embodiments, each incoming droplet of first sample fluid is merged with the same amount of second sample fluid.

In certain embodiments, an electric charge is applied to the first and second sample fluids. Description of applying electric charge to sample fluids is provided in Link et al. (U.S. patent application number 2007/0003442) and European Patent Number EP2004316 to Raindance Technologies Inc, the content of each of which is incorporated by reference herein in its entirety. Electric charge may be created in the first and second sample fluids within the carrier fluid using any suitable technique, for example, by placing the first and second sample fluids within an electric field (which may be AC, DC, etc.), and/or causing a reaction to occur that causes the first and second sample fluids to have an electric charge, for example, a chemical reaction, an ionic reaction, a photocatalyzed reaction, etc.

The electric field, in some embodiments, is generated from an electric field generator, i.e., a device or system able to create an electric field that can be applied to the fluid. The electric field generator may produce an AC field (i.e., one that varies periodically with respect to time, for example, sinusoidally, sawtooth, square, etc.), a DC field (i.e., one that is constant with respect to time), a pulsed field, etc. The electric field generator may be constructed and arranged to create an electric field within a fluid contained within a channel or a microfluidic channel. The electric field generator may be integral to or separate from the fluidic system containing the channel or microfluidic channel, according to some embodiments.

Techniques for producing a suitable electric field (which may be AC, DC, etc.) are known to those of ordinary skill in the art. For example, in one embodiment, an electric field is produced by applying voltage across a pair of electrodes, which may be positioned on or embedded within the fluidic system (for example, within a substrate

defining the channel or microfluidic channel), and/or positioned proximate the fluid such that at least a portion of the electric field interacts with the fluid. The electrodes can be fashioned from any suitable electrode material or materials known to those of ordinary skill in the art, including, but not limited to, silver, gold, copper, carbon, platinum, copper, tungsten, tin, cadmium, nickel, indium tin oxide ("ITO"), etc., as well as combinations thereof. In some cases, transparent or substantially transparent electrodes can be used.

The electric field facilitates rupture of the interface separating the second sample fluid and the droplet. Rupturing the interface facilitates merging of bolus of the second sample fluid and the first sample fluid droplet. The forming mixed droplet continues to increase in size until it a portion of the second sample fluid breaks free or segments from the second sample fluid stream prior to arrival and merging of the next droplet containing the first sample fluid. The segmenting of the portion of the second sample fluid from the second sample fluid stream occurs as soon as the shear force exerted on the forming mixed droplet by the immiscible carrier fluid overcomes the surface tension whose action is to keep the segmenting portion of the second sample fluid connected with the second sample fluid stream. The now fully formed mixed droplet continues to flow through the first channel.

In other embodiments, the rupture of the interface can be spontaneous, or the rupture can be facilitated by surface chemistry. The invention is not limited in regard to the method of rupture at the interface, as rupture can be brought about by any means.

In the context of PCR, in a preferred embodiment, the first sample fluid contains nucleic acid templates. Droplets of the first sample fluid are formed as described above. Those droplets will include the nucleic acid templates. In certain embodiments, the droplets will include only a single nucleic acid template, and thus digital PCR can be conducted. The second sample fluid contains reagents for the PCR reaction. Such reagents generally include Taq polymerase, deoxynucleotides of type A, C, G and T, magnesium chloride, and forward and reverse primers, all suspended within an aqueous buffer. The second fluid also includes detectably labeled probes for detection of the amplified target nucleic acid, the details of which are discussed below. A droplet containing the nucleic acid is then caused to merge with the PCR reagents in the second fluid as described above, producing a droplet that includes Taq polymerase, deoxynucleotides of type A, C, G and T, magnesium chloride, forward and reverse primers, detectably labeled probes, and the target nucleic acid. In another embodiment, the first fluid can contain the template DNA and PCR master mix (defined below), and the second fluid can contain the forward and reverse primers and the probe. The invention is not restricted in any way regarding the constituency of the first and second fluids for PCR or digital PCR. For example, in some embodiments, the template DNA is contained in the second fluid inside droplets.

Target Amplification

Methods of the invention further involve amplifying the target nucleic acid in each droplet. Amplification refers to production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction or other technologies well known in the art (e.g., Dieffenbach and Dveksler, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y. [1995]). The amplification reaction may be any amplification reaction known in the art that amplifies nucleic acid molecules, such as polymerase chain reaction, nested polymerase chain reaction, ligase chain reaction (Barany F. (1991) PNAS 88:189-193; Barany F. (1991) PCR Methods and Applications 1:5-16), ligase detection reaction (Barany F. (1991) PNAS 88:189-193), strand displacement amplification, transcription based amplification system, nucleic acid sequence-based amplification, rolling circle amplification, and hyper-branched rolling circle amplification.

In certain embodiments, the amplification reaction is the polymerase chain reaction. Polymerase chain reaction (PCR) refers to methods by K. B. Mullis (U.S. Pat. Nos. 4,683,195 and 4,683,202, hereby incorporated by reference) for increasing concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. The process for amplifying the target sequence includes introducing an excess of oligonucleotide primers to a DNA mixture containing a desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The primers are complementary to their respective strands of the double stranded target sequence.

To effect amplification, primers are annealed to their complementary sequence within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one cycle; there can be numerous cycles) to obtain a high concentration of an amplified segment of a desired target sequence. The length of the amplified segment of the desired target sequence is determined by relative positions of the primers with respect to each other and by cycling parameters, and therefore, this length is a controllable parameter.

Methods for performing PCR in droplets are shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Anderson et al. (U.S. Pat. No. 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc. The content of each of which is incorporated by reference herein in its entirety.

The sample droplet may be pre-mixed with a primer or primers, or the primer or primers may be added to the droplet. In some embodiments, droplets created by segmenting the starting sample are merged with a second set of droplets including one or more primers for the target nucleic acid in order to produce final droplets. The merging of droplets can be accomplished using, for example, one or more droplet merging techniques described for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

In embodiments involving merging of droplets, two droplet formation modules are used. In one embodiment, a first droplet formation module produces the sample droplets consistent with limiting or terminal dilution of target nucleic acid. A second droplet formation or reinjection module inserts droplets that contain reagents for a PCR reaction. Such droplets generally include the "PCR master mix" (known to those in the art as a mixture containing at least Taq polymerase, deoxynucleotides of type A, C, G and T, and magnesium chloride) and forward and reverse primers (known to those in the art collectively as "primers"), all suspended within an aqueous buffer. The second droplet also includes detectably labeled probes for detection of the amplified target nucleic acid, the details of which are discussed below. Different arrangements of reagents between the two droplet types is envisioned. For example, in another embodiment, the template droplets also contain the PCR master mix, but the primers and probes remain in the second droplets. Any arrangement of reagents and template DNA can be used according to the invention.

Primers can be prepared by a variety of methods including but not limited to cloning of appropriate sequences and direct chemical synthesis using methods well known in the art (Narang et al., *Methods Enzymol.*, 68:90 (1979); Brown et al., *Methods Enzymol.*, 68:109 (1979)). Primers can also be obtained from commercial sources such as Operon Technologies, Amersham Pharmacia Biotech, Sigma, and Life Technologies. The

primers can have an identical melting temperature. The lengths of the primers can be extended or shortened at the 5' end or the 3' end to produce primers with desired melting temperatures. Also, the annealing position of each primer pair can be designed such that the sequence and, length of the primer pairs yield the desired melting temperature. The simplest equation for determining the melting temperature of primers smaller than 25 base pairs is the Wallace Rule ($T_d = 2(A+T) + 4(G+C)$). Another method for determining the melting temperature of primers is the nearest neighbor method. Computer programs can also be used to design primers, including but not limited to Array Designer Software (Arrayit Inc.), Oligonucleotide Probe Sequence Design Software for Genetic Analysis (Olympus Optical Co.), NetPrimer, and DNAs is from Hitachi Software Engineering. The T_m (melting or annealing temperature) of each primer is calculated using software programs such as Oligo Design, available from Invitrogen Corp.

In one embodiment, the droplet formation modules are arranged and controlled to produce an interdigitation of sample droplets and PCR reagent droplets flowing through a channel. Such an arrangement is described for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

A sample droplet is then caused to merge with a PCR reagent droplet, producing a droplet that includes the PCR master mix, primers, detectably labeled probes, and the target nucleic acid. Droplets may be merged for example by: producing dielectrophoretic forces on the droplets using electric field gradients and then controlling the forces to cause the droplets to merge; producing droplets of different sizes that thus travel at different velocities, which causes the droplets to merge; and producing droplets having different viscosities that thus travel at different velocities, which causes the droplets to merge with each other. Each of those techniques is further described in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc. Further description of producing and controlling dielectrophoretic forces on droplets to cause the droplets to merge is described in Link et al. (U.S. patent application number 2007/0003442) and European Patent Number EP2004316 to Raindance Technologies Inc.

In another embodiment, called simple droplet generation, a single droplet formation module, or a plurality of droplet formation modules are arranged to produce droplets from a mixture already containing the template DNA, the PCR master mix, primers, and detectably labeled probes. In yet another embodiment, called co-flow, upstream from a single droplet formation module two channels intersect allowing two flow streams to converge. One flow stream contains one set of reagents and the template DNA, and the other contains the remaining reagents. In the preferred embodiment for co-flow, the template DNA and the PCR master mix are in one flow stream, and the primers and probes are in the other. However, the invention is not limited in regard to the constituency of either flow stream. For example, in another embodiment, one flow stream contains just the template DNA, and the other contains the PCR master mix, the primers, and the probes. On convergence of the flow streams in a fluidic intersection, the flow streams may or may not mix before the droplet generation nozzle. In either embodiment, some amount of fluid from the first stream, and some amount of fluid from the second stream are encapsulated within a single droplet. Following encapsulation, complete mixing occurs.

Once final droplets have been produced by any of the droplet forming embodiments above, or by any other embodiments, the droplets are thermal cycled, resulting in amplification of the target nucleic acid in each droplet. In certain embodiments, the droplets are collected off-chip as an emulsion in a PCR thermal cycling tube and then thermally cycled in a conventional thermal cycler. Temperature profiles for thermal cycling can be adjusted

and optimized as with any conventional DNA amplification by PCR.

In certain embodiments, the droplets are flowed through a channel in a serpentine path between heating and cooling lines to amplify the nucleic acid in the droplet. The width and depth of the channel may be adjusted to set the residence time at each temperature, which can be controlled to anywhere between less than a second and minutes.

In certain embodiments, the three temperature zones are used for the amplification reaction. The three temperature zones are controlled to result in denaturation of double stranded nucleic acid (high temperature zone), annealing of primers (low temperature zones), and amplification of single stranded nucleic acid to produce double stranded nucleic acids (intermediate temperature zones). The temperatures within these zones fall within ranges well known in the art for conducting PCR reactions. See for example, Sambrook et al. (Molecular Cloning, A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).

In certain embodiments, the three temperature zones are controlled to have temperatures as follows: 95.degree. C. (T.sub.H), 55.degree. C. (T.sub.L), 72.degree. C. (T.sub.M). The prepared sample droplets flow through the channel at a controlled rate. The sample droplets first pass the initial denaturation zone (T.sub.H) before thermal cycling. The initial preheat is an extended zone to ensure that nucleic acids within the sample droplet have denatured successfully before thermal cycling. The requirement for a preheat zone and the length of denaturation time required is dependent on the chemistry being used in the reaction. The samples pass into the high temperature zone, of approximately 95.degree. C., where the sample is first separated into single stranded DNA in a process called denaturation. The sample then flows to the low temperature, of approximately 55.degree. C., where the hybridization process takes place, during which the primers anneal to the complementary sequences of the sample. Finally, as the sample flows through the third medium temperature, of approximately 72.degree. C., the polymerase process occurs when the primers are extended along the single strand of DNA with a thermostable enzyme. Methods for controlling the temperature in each zone may include but are not limited to electrical resistance, peltier junction, microwave radiation, and illumination with infrared radiation.

The nucleic acids undergo the same thermal cycling and chemical reaction as the droplets passes through each thermal cycle as they flow through the channel. The total number of cycles in the device is easily altered by an extension of thermal zones or by the creation of a continuous loop structure. The sample undergoes the same thermal cycling and chemical reaction as it passes through N amplification cycles of the complete thermal device.

In other embodiments, the temperature zones are controlled to achieve two individual temperature zones for a PCR reaction. In certain embodiments, the two temperature zones are controlled to have temperatures as follows: 95.degree. C. (T.sub.H) and 60.degree. C. (T.sub.L). The sample droplet optionally flows through an initial preheat zone before entering thermal cycling. The preheat zone may be important for some chemistry for activation and also to ensure that double stranded nucleic acid in the droplets are fully denatured before the thermal cycling reaction begins. In an exemplary embodiment, the preheat dwell length results in approximately 10 minutes preheat of the droplets at the higher temperature.

The sample droplet continues into the high temperature zone, of approximately 95.degree. C., where the sample is first separated into single stranded DNA in a process called denaturation. The sample then flows through the device to the low temperature zone, of approximately 60.degree. C., where the hybridization process takes

place, during which the primers anneal to the complementary sequences of the sample. Finally the polymerase process occurs when the primers are extended along the single strand of DNA with a thermostable enzyme. The sample undergoes the same thermal cycling and chemical reaction as it passes through each thermal cycle of the complete device. The total number of cycles in the device is easily altered by an extension of block length and tubing.

In another embodiment the droplets are created and/or merged on chip followed by their storage either on the same chip or another chip or off chip in some type of storage vessel such as a PCR tube. The chip or storage vessel containing the droplets is then cycled in its entirety to achieve the desired PCR heating and cooling cycles.

In another embodiment the droplets are collected in a chamber where the density difference between the droplets and the surrounding oil allows for the oil to be rapidly exchanged without removing the droplets. The temperature of the droplets can then be rapidly changed by exchange of the oil in the vessel for oil of a different temperature. This technique is broadly useful with two and three step temperature cycling or any other sequence of temperatures.

The invention is not limited by the method used to thermocycle the droplets. Any method of thermocycling the droplets may be used.

Target Detection

After amplification, droplets are flowed to a detection module for detection of amplification products. For embodiments in which the droplets are thermally cycled off-chip, the droplets require re-injection into either a second fluidic circuit for read-out--that may or may not reside on the same chip as the fluidic circuit or circuits for droplet generation--or in certain embodiments the droplets may be reinjected for read-out back into the original fluidic circuit used for droplet generation. The droplets may be individually analyzed and detected using any methods known in the art, such as detecting the presence or amount of a reporter. Generally, the detection module is in communication with one or more detection apparatuses. The detection apparatuses can be optical or electrical detectors or combinations thereof. Examples of suitable detection apparatuses include optical waveguides, microscopes, diodes, light stimulating devices, (e.g., lasers), photo multiplier tubes, and processors (e.g., computers and software), and combinations thereof, which cooperate to detect a signal representative of a characteristic, marker, or reporter, and to determine and direct the measurement or the sorting action at a sorting module. Further description of detection modules and methods of detecting amplification products in droplets are shown in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

In certain embodiments, amplified target are detected using detectably labeled probes. In particular embodiments, the detectably labeled probes are optically labeled probes, such as fluorescently labeled probes. Examples of fluorescent labels include, but are not limited to, Atto dyes, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; acridine and derivatives: acridine, acridine isothiocyanate; 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl]phenyl]naphthalimide-3,5-disulfonate; N-(4-anilino-1-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives; coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcoumarin (Coumarin 151); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5'5'-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin;

diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives; eosin, eosin isothiocyanate, erythrosin and derivatives; erythrosin B, erythrosin, isothiocyanate; ethidium; fluorescein and derivatives; 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF), 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, QFITC, (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferoneortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives: pyrene, pyrene butyrate, succinimidyl 1-pyrene; butyrate quantum dots; Reactive Red 4 (Cibacron.TM. Brilliant Red 3B-A) rhodamine and derivatives: 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid; terbium chelate derivatives; Cy3; Cy5; Cy5.5; Cy7; IRD 700; IRD 800; La Jolla Blue; phthalocyanine; and naphthalocyanine. Preferred fluorescent labels are FAM and VIC.TM. (from Applied Biosystems). Labels other than fluorescent labels are contemplated by the invention, including other optically-detectable labels.

In certain aspects, the droplets of the invention contain a plurality of detectable probes that hybridize to amplicons produced in the droplets. Members of the plurality of probes can each include the same detectable label, or a different detectable label. The plurality of probes can also include one or more groups of probes at varying concentration. The groups of probes at varying concentrations can include the same detectable label which vary in intensity, due to varying probe concentrations.

In a separate embodiment the detection can occur by the scanning of droplets confined to a monolayer in a storage device that is transparent to the wavelengths or method of detection. Droplets stored in this fashion can be scanned either by the movement of the storage device by the scanner or the movement of the scanner over the storage device.

The invention is not limited to the TaqMan assay, as described above, but rather the invention encompasses the use of all fluorogenic DNA hybridization probes, such as molecular beacons, Solaris probes, scorpion probes, and any other probes that function by sequence specific recognition of target DNA by hybridization and result in increased fluorescence on amplification of the target sequence.

Digital PCR Performance in Droplets

Digital PCR performance in the emulsion format was validated by measuring a serial dilution of a reference gene, branched chain keto acid dehydrogenase E1 (BCKDHA). Mixtures of the PCR master mix, 1.times. primers and probe for BCKDHA, and varying concentrations of a mixture of human genomic DNA (1:1 NA14091 and NA13705) were compartmentalized into over one million 5.3 pL droplets in a water-in-fluorinated oil emulsion using the droplet generation microfluidic chip. The emulsion was thermally cycled off-chip and afterwards the fluorescence of each droplet was analyzed by fluorescence in the readout chip (see FIG. 3).

An exemplary microfluidic system for droplet generation and readout is depicted in FIG. 3. The microfluidic system for droplet generation and readout. As shown in FIG. 3a (droplet generation chip), a continuous aqueous

phase containing the PCR master mix, primers, and probes, and template DNA flowed into the fluidic intersection from the left, and the carrier oil entered from the top and bottom. An emerging bolus of aqueous liquid was imaged inside the intersection just prior to snapping off into a discrete 4 pL droplet as the fluidic strain began to exceed the surface tension of the aqueous liquid. The steady train of droplets leaving the intersection toward the right was collected off chip as a stable emulsion for thermal cycling. FIG. 3b depicts the droplet spacing for readout. Flows were arranged as in 3a, except instead of a continuous phase, the emulsion from (a) was injected from the left into the intersection after thermal cycling. The oil drained from the emulsion during off-chip handling, hence the emulsion appeared tightly packed in the image before the intersection. The oil introduced in the intersection separated the droplets and the fluorescence of each droplet was measured at the location marked by the arrow. FIG. 3c depicts a cartoon of droplet readout by fluorescence. The relatively infrequent PCR(+) droplets (light gray) flow along with the majority of PCR(-) droplets (dark gray) toward the detector. The droplets were interrogated sequentially by laser induced fluorescence while passing through the detection region.

In a serial dilution the average number of target DNA molecules per droplet--called the "occupancy" from this point forward--should decrease in direct proportion to the DNA concentration. The occupancy was calculated from Poisson statistics using the following equation well known to those experienced in the art:

.function. ##EQU00001## where P and N are the numbers of PCR(+) and PCR(-) droplets respectively.

Droplets were analyzed by fluorescence while flowing through the readout chip to count the numbers of PCR(+) and PCR(-) droplets (see FIG. 3c). As each droplet passed the detection zone (marked with an arrow in FIG. 3b), a burst of fluorescence was observed. To account for small run-to-run differences in the fluorescence intensity that can occur due to different chip positioning, etc., each set of data was scaled such that the average fluorescence intensity of the empty droplets was 0.1 V. FIG. 4a shows a very short duration of a typical trace of fluorescence bursts from individual droplets for the sample with the highest DNA concentration in the series. PCR(+) and PCR(-) droplets were easily discriminated by fluorescence intensity. The two large bursts of fluorescence peaking at .about.0.8 V arose from the PCR(+) droplets, whereas the smaller bursts due to incomplete fluorescence quenching in the PCR(-) droplets peaked at .about.0.1 V. A histogram of peak intensities from the complete data set revealed two clear populations centered at 0.10 and 0.78 V (FIG. 4b), demonstrating that the trend evident in the short trace in FIG. 4a was stable over much longer periods of time. Integration over the two populations in FIG. 4b yielded a total of 197,507 PCR(+) and 1,240,126 PCR(-) droplets. Hence the occupancy was 0.15 for this sample by Eqn. 1, corresponding to the expected occupancy of 0.18 based on the measured DNA concentration of 110 ng/.mu.L. The occupancy was measured for each sample in the serial dilution and fit to the dilution equation:

.function. ##EQU00002## where n is the number of dilutions, A is the occupancy at the starting concentration (n=0), and f is the dilution factor. The linear fit was in excellent agreement with the data, with an R.sup.2 value of 0.9999 and the fitted dilution factor of 4.8 in close agreement with the expected value of 5.0. Multiplexing Primers in a Digital PCR Reaction

Droplet based digital PCR technology, as described in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163), Anderson et al. (U.S. Pat. No. 7,041,481 and which reissued as RE41,780) and European publication number EP2047910 to Raindance Technologies Inc, (the contents of each of which are incorporated by reference herein in their entireties) utilizes a single primer pair per

library droplet. This library droplet is merged with a template droplet which contains all the PCR reagents including genomic DNA except for the primers. After merging of the template and the primer library droplets the new droplet now contains all the reagents necessary to perform PCR. The droplet is then thermal cycled to produce amplicons. In one embodiment, the template DNA is diluted in the template mix such that on average there is less than one haploid genome per droplet.

Having only one haploid genome (i.e., one allele) per droplet gives droplet PCR advantages over standard singleplex or multiplex PCR in tubes or microwells. For example, in traditional PCR, both alleles are present in the reaction mix so if there is a difference in the PCR efficiency between alleles, the allele with the highest efficiency will be over represented. Additionally, there can be variances in the sequence to which the PCR primers hybridize, despite careful primer design. A variance in the primer hybridization sequence can cause that primer to have a lower efficiency for hybridization for the allele that has the variance compared to the allele that has the wild type sequence. This can also cause one allele to be amplified preferentially over the other allele if both alleles are present in the same reaction mix.

These issues are avoided in droplet based PCR because there is only one template molecule per droplet, and thus one allele per droplet. Thus, even if primer variance exists that reduces the PCR efficiency for one allele, there is no competition between alleles because the alleles are separated and thus uniformly amplified.

Optimization of traditional multiplexing of standard PCR primers in tubes or wells is known to be difficult. Multiple PCR amplicons being generated in the same reaction can lead to competition between amplicons that have differing efficiencies due to differences in sequence or length. This results in varying yields between competing amplicons which can result in non uniform amplicon yields. However, because droplet based digital PCR utilizes only one template molecule per droplet, even if there are multiple PCR primer pairs present in the droplet, only one primer pair will be active. Since only one amplicon is being generated per droplet, there is no competition between amplicons, resulting in a more uniform amplicon yield between different amplicons.

A certain amount of DNA is required to generate either a specific quantity of DNA and/or a specific number of PCR positive droplets to achieve sufficient sequencing coverage per base. Because only a percentage of the droplets are PCR positive, approximately 1 in 3 in the standard procedure, it takes more DNA to achieve the equivalent PCR yield per template DNA molecule. The number of PCR positive droplets and thus the amplicon yield can be increased by adding more genomic DNA. For instance, increasing the amount of genomic DNA twofold while maintaining the number of droplets constant will double the amplicon yield. However there is a limit to the amount of genomic DNA that can be added before there is a significant chance of having both alleles for a gene in the same droplet, thereby eliminating the advantage of droplet PCR for overcoming allele specific PCR and resulting in allelic dropout.

One way to allow the input of more genomic DNA is by generating more droplets to keep the haploid molecules per droplet ratio constant. For instance doubling the amount of DNA and doubling the amount of droplets increases the amplicon yield by 2.times. while maintaining the same haploid genome per droplet ratio. However, while doubling the number of droplets isn't problematic, increasing the amount of DNA can be challenging to users that have a limited amount of DNA.

The multiplexing of PCR primers in droplets enables the simultaneous increase in the number of PCR droplets while keeping the amount of input DNA the same or lower to generate an equal or greater amplicon yield. This

results in an overall increase in the amount of PCR positive droplets and amplicon yield without the consumption of more DNA.

By way of example, if there is an average of 1 haploid genome per every 4 droplets or 1/4 of the haploid genome per droplet and one PCR primer pair per droplet, the chances of the correct template being present for the PCR primer in the droplet is 1 out of 4. However, if there are 2 PCR primer pairs per droplet, then there is double the chance that there will be the correct template present in the droplet. This results in 1 out of 2 droplets being PCR positive which doubles the amplicon yield without doubling the input DNA. If the number of droplets containing the 2.times. multiplexed primers is doubled and the DNA kept constant, then the number of PCR positive droplets drops back to 1 in 4, but the total number of PCR droplets remains the same because the number of droplets have been doubled. If the multiplexing level in each droplet is increased to 4.times. and the input DNA is the same, the chance of the correct template molecule being present in each droplet doubles. This results in the number of PCR positive droplets being increased to 1 in 2 which doubles the amount of amplicon yield without increasing the amount of input DNA. Thus, by increasing the multiplexing of PCR primers in each droplet and by increasing the number of droplets overall, the amplicon yield can be increased by 4-fold without increasing the amount of input DNA.

Alternatively, if the amplicon yield is already sufficient, by increasing the multiplexing level for the PCR primers in each droplet, the amount of input genomic DNA can be dropped without sacrificing amplicon yield. For example if the multiplexing level of the PCR primers goes from 1.times. to 2.times., the amount of input genomic DNA can be decreased by 2.times. while still maintaining the same overall amplicon yield.

Even though the number of PCR primer pairs per droplet is greater than one, there is still only one template molecule per droplet and thus there is only one primer pair per droplet that is being utilized at one time. This means that the advantages of droplet PCR for eliminating bias from either allele specific PCR or competition between different amplicons is maintained.

An example demonstration of droplet-based amplification and detection of multiple target sequences in a single droplet is shown here. Multiple copies of 5 sets of primers (primers for TERT, RNaseP, E1a, SMN1 and SMN2) were encapsulated in a single droplet at various concentrations along with the template DNA and the PCR master mix. Probes that specifically bind to TERT, RNaseP, E1a, SMN1 or SMN2 were also encapsulated in the droplets containing the primers. Probes for TERT, RNaseP and E1a were labeled with the VIC dye and probes for SMN1 and SMN2 were labeled with the FAM dye. The sequences for TERT, RNaseP, E1a, SMN1 and SMN2 were amplified by PCR. The PCR was conducted with a standard thermal cycling setting. For example: 95.degree. C. for 10 min 31 cycles 92.degree. C. for 15 s 60.degree. C. for 60 s

At the end of the PCR, the fluorescence emission from each droplet was determined and plotted on a scattered plot based on its wavelength and intensity. Six clusters, each representing droplets having the corresponding fluorescence wavelength and intensity were shown. The TERT, RNaseP and E1a clusters showed the fluorescence of the VIC dye at three distinct intensities and SMN1 and SMN2 clusters showed the fluorescence of the FAM dye at two distinct intensities (FIG. 5). The number of droplets, each having one or more sequences selected from TERT, RNaseP, E1a, SMN1 and SMN2, can be determined from the scattered plot.

In another demonstration of droplet-based amplification and detection of multiple target sequences in a single droplet, 5 sets of primers (primers for TERT, RNaseP, E1a, 815A and 815G) were encapsulated in a single

droplet at various concentrations along with the template DNA, the PCR master mix, and the probes. The five different probes TERT, RNaseP, E1a, 815A and 815G were also encapsulated in the droplets containing the primers. Probes for TERT and 815A were labeled with the VIC dye and probes for 815G were labeled with the FAM dye. For each of RNaseP and E1a, two probes, one labeled with the VIC dye and the other labeled with the FAM dye, were encapsulated.

The droplets containing both the primers and probes were fused with droplets containing the template. PCR reactions were conducted with the fused droplets to amplify the sequences for TERT, RNaseP, E1a, 815A and 815G. The PCR was conducted with a standard thermal cycling setting.

At the end of the PCR, the fluorescence emission from each fused droplet was determined and plotted on a scattered plot based on its wavelength and intensity. Six clusters, each representing droplets having the corresponding fluorescence wavelength and intensity were shown. The TERT and 815A clusters showed the fluorescence of the VIC dye at two distinct intensities; the 815G clusters showed the fluorescence of the FAM dye; and the RNaseP and E1a clusters showed the fluorescence of both the FAM and the VIC dye at distinct intensities (FIG. 6). The number of droplets, each having one or more sequences selected from TERT, RNaseP, E1a, 815A and 815G, can be determined from the scattered plot. The copy number of RNaseP, E1a, 815A and 815G in the template were determined by the ratio between the number of droplets having the RNaseP, E1a, 815A and/or 815G sequences and the number of droplets having the TERT sequence (FIG. 6).

In yet another exemplary demonstration of multiplexed primer pairs in a droplet-based digital PCR reaction, two droplet libraries were generated: droplet library A was generated where each droplet contained only one primer pair; and droplet library B was generated where the primer pairs were multiplexed at 5.times. level in each droplet. HapMap sample NA18858 was processed in duplicate with droplet libraries A or B using standard procedures. Two .mu.g sample DNA was used for droplet library A and one .mu.g sample DNA was used for the 5.times. multiplex droplet library B. After PCR amplification, both droplet libraries were broken and purified over a Qiagen MinElute column and then run on an Agilent Bioanalyzer. Samples were sequenced by Illumina on the Illumina GAII with 50 nucleotide reads and the sequencing results were analyzed using the standard sequencing metrics. The results from the 5.times. multiplexed droplet library B were compared to the singleplex droplet library A using standard metrics shown in the Table below.

The results obtained from the 5.times. multiplexed droplet library B were equivalent or better than what was obtained from droplet library A. The multiplexing of primers delivers the same sequencing results for base coverage, specificity and uniformity that the singleplexing does with the added advantage of reduced input DNA.

TABLE-US-00001 Total Mapped Mean base Base coverage Sample reads reads Specificity coverage C1 C20
C100 (0.2.times. of mean) Library A 27431697 99.4% 0.813 1394 99.5% 99.0% 98.2% 92.8% with sample
1 Library A 15147288 99.4% 0.862 819 99.1% 98.2% 87.6% 78.0% with sample 2 Library B 27861378
99.5% 0.847 1472 99.7% 99.3% 97.6% 89.9% with sample 1 Library B 25758406 99.1% 0.837 1321
99.8% 99.4% 97.9% 91.3% with sample 2 Total reads: total number of sequencing read found within the
provided sample data. Mapped reads (%): percentage of total reads that mapped to the human genome.
Specificity: percentage of mapped reads that include the target. The target includes all amplicon sequences with
primer sequences excluded. Mean base coverage: average base coverage within the target. The target includes
all amplicon sequences with primer sequences excluded. C1: % of target that has at least 1.times. base coverage.
Note: non-unique sequencing reads are mapped randomly. C20: % of target that has at least 20.times. base

coverage. C100: % of target that has at least 100.times. base coverage. Base coverage (0.2.times. of mean): % of target that has at least 20% of mean base coverage.

Monochromatic Gene Copy Number Assay

Traditional digital PCR methods involve the use of a single labeled probe specific for an individual target. FIG. 7 is a schematic depicting one-color detection of a target sequence using droplet based digital PCR. As shown in Panel A of FIG. 7, a template DNA is amplified with a forward primer (F1) and a reverse primer (R1). Probe (P1) labeled with a fluorophore of color 1 binds to the target genetic sequence (target 1). Microdroplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Droplets containing the target sequence emit fluorescence and are detected by laser (Panels B and C). The number of microcapsules either containing or not containing the target sequence is shown in a histogram (D) and quantified (E).

FIG. 8 is a schematic depicting two-color detection of two genetic sequences with a microfluidic device. As shown in Panel A of FIG. 8, a template DNA is amplified with two sets of primers: forward primer (F1) and a reverse primer (R1), and forward primer (F2) and a reverse primer (R2). Probe (P1) labeled with a fluorophore of color 1 binds to the target 1 and probe (P2) labeled with a fluorophore of color 2 binds to the target 2 (Panels B and C). Droplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Droplets containing the target sequence 1 or 2 emit fluorescence of color 1 or 2 respectively and are optically detected by laser (Panels B and C). The number of microcapsules containing target 1 or 2 is shown by histogram in Panel D.

Methods of the invention involve performing accurate quantitation of multiple different DNA targets by dPCR using probes with the same fluorophore. FIG. 9 is a schematic depicting two-color detection of three genetic sequences with a microfluidic device. As shown in Panel A of FIG. 9, a template DNA is amplified with three sets of primers: forward primers (F1, F2 and F3) and reverse primers (R1, R2 and R3). Probes (P1, P2 and P3) are labeled with fluorophores (color 1, color 2 and color 1) and bind to the target genetic sequences (target 1, target 2 and target 3) (Panels B and C). Microdroplets are made of diluted solution of template DNA under conditions of limiting or terminal dilution. Microdroplets containing target sequence 1 or 3 emit fluorescence of color 1 at two different intensities; and microdroplets containing target sequence 2 emit fluorescence of color 2. The number of microdroplets containing target 1, 2 or 3 is shown by histogram in Panel D.

Recent results from the droplet digital PCR (dPCR) shows that multiple independent PCR reactions can be run and separately quantified using the same fluorophore. Specifically, an SMN2 assay yields an unexpected population of droplets with slightly elevated signal in the FAM detection channel.

The results are depicted in FIG. 10. The left-side dot plot in FIG. 10 depicts the effect of having the SMN1 blocker present in the reaction. The four clusters depicted in the left-side dot plot are as follows: the top left cluster includes microdroplets containing the reference sequence (SMARCC1); the bottom left cluster includes microdroplets not containing any sequence; the bottom middle cluster includes microdroplets containing sequence for SMN1; and the bottom right cluster includes microdroplets containing sequence for SMN2. The dot plot on the right-side of FIG. 10 depicts four clusters where no SMN1 blocker was present in the reaction: the top left cluster includes microdroplets containing the reference sequence (SMARCC1); the bottom left cluster includes microdroplets not containing any sequence; the bottom middle cluster includes microdroplets containing sequence for SMN1; and the bottom right cluster includes microdroplets containing sequence for

SMN2. The shift of the bottom middle cluster in right panel as compared to left panel confirms that fluorescence intensity provides a very sensitive measurement for the presence of a sequence.

Without intending to be bound by any theory, the simplest explanation is that the cluster arises from weak association of the SMN2 probe to the SMN1 gene despite the presence of a blocker to that gene (a nonfluorescent complementary probe to the SMN1 gene).

One definitive confirmation of SMN1 as the source of the unexpected cluster was an observed dependence of the intensity of this feature on the presence of the SMN1 blocker. A clear shift toward higher FAM fluorescent intensities was observed in the absence of the blocker (FIG. 10). In another definitive confirmation the ratio of the SMN1 (putative) population size to the reference size of 0.96 in perfect agreement with expectation (two copies of each) (S_131 sample). Another sample, S_122, with the same number of SMN1 copies yielded a ratio of 0.88 in one run and 0.93 in another, also consistent with the proposed explanation of the unexpected cluster.

Without intending to be bound by any theory, these observations indicate that SMN2 probe binding to SMN1 DNA yields an elevated fluorescent signal. A simple kinetic model explaining this phenomenon assumes that the hybridization of the SMN2 probe to the SMN1 DNA achieves equilibrium at a faster rate than the polymerase fills in the complementary strand. The amount of probe fluorophore that is released in each thermal cycle is therefore proportional to (or even equal to) the number of bound probes. Thus the lower the binding affinity the fewer the number of probe fluorophores that are released. Due to SMN1 sequence mismatch(es) with the SMN2 probe, the affinity of the probe is certainly expected to be lower to SMN1 than SMN2. This model also explains the signal dependence on the SMN1 blocker: the blocker competitively inhibits the SMN2 probe hydrolysis by the polymerase exonuclease activity.

It may also be, however, that the probe hybridization does not reach equilibrium before exonuclease activity. In this case, the association rates would play a more dominant role. Similar logic applies. The binding rate to the matching site is likely to be faster than to the mismatch site, and the blocker would act to decelerate probe binding to the mismatch site. The binding of SMN2 probe to SMN1 DNA might be detectable by conventional bulk qPCR, especially in absence of SMN2, but highly quantitative results like those shown here are very unlikely. Definitely, there is no report of qPCR or any other technique quantifying two different DNA sequence motifs with the same color fluorophore. Sequestration of the individual reactions by single molecule amplification within droplets eliminates any confusion regarding mixed contributions to the signal.

The advantage of quantifying DNA with multiple probes of the same color fluorophore extends beyond the example of two highly homologous sequences shown here. Rather, any plurality of sequences of any degree of similarity or dissimilarity can be quantified so long as the different probes have significantly different binding occupancies to their respective DNA binding sites.

Another advantage of the dPCR approach for multiplexed reactions is that the different reactions do not compete with each other for reagents as they would in a bulk qPCR assay. However, the possibility for unintended cross-reactivity remains. A multiplexed assay can require a more dilute sample. For instance, at 10% occupancy a duplex reaction would have double occupancy 1% of the time. Hence 1 in 10 PCR+ droplets would be doubles, resulting in a final intensity at least as high and possibly higher than the brighter of the two probes. For a simple duplex system the contribution from each probe could be recovered. In this example the total number of PCR+

droplets for probe 1 would be (Probe 1)+(Probe 1+Probe 2). Higher degrees of multiplexing would require greater dilution. For example, for a 4-plex at 1% occupancy the probability of one probe overlapping any of the other 3 is .about.3%, and that error may be too high for some applications. The need for large dilutions strongly favors the large number of dPCR reactions.

In another example of the invention, a single fluorophore (FAM) was used in a gene copy number assay for both the reference and the target DNA. A model system was used with varying concentrations of plasmid DNA to represent a change in the target gene copy number, relative to a reference gene, equivalent to 0-16 copies of the target gene per cell. BCKDHA and SMN2 plasmid DNA served as the reference and target with 1.times. and 0.5.times. primers and probes respectively. With a starting ratio of 8:1 SMN2 to BCKDHA, the sample was diluted serially by 2.times. into a solution of BCKDHA at the same concentration to vary just the amount of SMN2. The resultant samples were emulsified, thermally cycled, and over 10.sup.5 droplets were analyzed for each sample as described in the previous section. The process was repeated in triplicate.

Methods of the invention also include analytical techniques for identification of fluorescence signatures unique to each probe. In this example of the invention, histograms of the droplet fluorescence intensities are shown in FIG. 11a for three different template DNA samples: a no template control (dotted line), BCKDHA only (solid line), and 1:1 BCKDHA to SMN2 (dashed line). For clarity, the histograms are shown both overlapped to highlight the similarity for certain peaks, and offset from each other to reveal all of the features. In the case of 1:1 BCKDHA to SMN2, three populations were readily apparent: a dominant feature appeared at 0.08 V, and two smaller peaks were evident at 0.27 and 0.71 V. The dominant feature at 0.08 V was assigned to PCR(-) droplets since both small peaks disappeared, but the large one remained, in the no template control. The peak at 0.71 V was assigned to BCKDHA since it was the sole feature arising with the addition of just BCKDHA, and the peak at 0.27 V appeared on subsequent addition of SMN2, completing the assignments. A very small peak appeared at .about.0.9 V, not visible on the scale of FIG. 11a, that corresponded to droplets occupied by both genes. As another method of the invention, once the different peaks are identified, droplets within each peak were counted corresponding to each possible state (PCR(+)) for either BCKDHA or SMN2, or both, or PCR(-)), and the gene copy number was then determined from the ratio of occupancies. Gene copy numbers for each sample in the serial dilution are plotted in FIG. 11b against expected values (observed ratios of SMN2 to BCKDHA to expected ratios of SMN2 to BSKDHA), with an excellent linear fit ($y=1.01x$) across the full range ($R_{sup.2}=0.9997$, slope=1.01), demonstrating accurate and precise measurement of the equivalent of 0 to 16 copies of SMN2 per cell.

Detection Of Alternatively Spliced Transcripts

The same principle can be used to detect and count alternatively spliced transcripts. TaqMan assays can be designed that are specific for each of the exons in an RNA transcript. After the RNA is turned into cDNA it can be encapsulated into a droplet at 1 copy or less per droplet. The droplet would also contain the multiplexed TaqMan assay for each of the exons. Each of the TaqMan assays would contain a different probe but all the probes would have the same fluorescent dye attached. The droplets would be thermocycled to generate signal for each of the TaqMan assays. If there are multiple splice variants in the sample they each will contain a different number of exons depending on the splicing events. The fluorescent intensity of each droplet would be different depending on the number of exons present. By counting the number of droplets with different intensities it would be possible to identify the presence and abundance of different splice variants in a sample.

Copy Number Variants In A Heterogeneous Sample

It would be possible to determine if a heterogeneous sample contained components with different copy level numbers. If the copy number variants to be assayed were spaced close enough along the chromosome, the DNA from a sample could be fragmented and encapsulated in droplets at a level of one haploid genomic equivalent or less per droplet. The droplet would also contain a TaqMan assay specific for the copy number variant. The intensity of the signal in each droplet would depend on the number of copy number variants are present for the sample. Counting of the number of droplets of different intensities would indicate things like how many cells in a particular sample had what level of copy number variants.

Tuning TaqMan.RTM. Probe Fluorescence Intensity

Identifying probes by fluorescence intensity often requires adjusting the brightness of the probes, particularly for higher-plex assays with dense probe patterns. In the previous section the probes for the gene copy number assay yielded very well resolved peaks (FIG. 11a). Clearly room exists to accommodate one or multiple extra probes in the copy number assay within the resolution of the measurement, but a method for adjusting the fluorescence intensity of the new probes is required to avoid interference with the existing assay. One method of the invention involves varying the probe and primer concentrations together as a very simple technique to optimize relative intensities in higher-plex reactions.

FIG. 12 is a schematic for tuning the intensity of a detectable label to a particular target with a microfluidic device. As shown in Panel A of FIG. 12, a template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2). Probes (P1 and P2) are labeled with fluorophore of color 1 and bind to target 1 and target 2 respectively. Fluorescence from target 2 is lower in intensity than that from target 1 due to single base mismatch between P2 and target 2. As shown in Panel B, template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2) (Panel B). Fluorescence from target 2 is lower in intensity than that from target 1 due to the presence of a competing probe 2 that is not labeled with the fluorophore. As shown in Panel C, template DNA is amplified with two sets of primers: forward primers (F1 and F2) and reverse primers (R1 and R2). Probes (P1 and P2) are labeled with fluorophore of color 1 and bind to target 1 and target 2 respectively. Fluorescence from target 2 is lower in intensity than that from target 1 due to the presence of a competing probe 2 that is labeled with a different fluorophore.

FIG. 13 shows probe fluorescence intensities throughout a serial dilution of the probes and primers for a different reference gene, ribonuclease P (RNaseP), against a constant amount of genomic DNA from the Coriell cell line NA3814 at an occupancy of 0.02 target DNA molecules per droplet. The probe fluorescent intensities varied in direct proportion to probe concentration over a narrow concentration range spanning about 0.15 to 0.4 μM ($R_{\text{sup.2}}=0.995$)--roughly centered about the typical probe concentration of 0.2 μM --after compensation for dilution errors and other run-to-run differences such as optical realignments using the intensity of the PCR(-) droplets as a reference. In summary, probe intensities can be varied by dilution over a small but adequate range for the purpose of tuning multiplexed assays without affecting the amplification itself.

Although the example above for adjusting probe fluorescence intensities involves varying probe and primer concentrations together by the same factor, the invention is not limited to this method alone for varying probe intensity. Other methods known to those familiar with the art for varying probe intensities are also considered. Such methods include varying just the probe concentration; varying just the primer concentrations; varying just

the forward primer concentration; varying just the reverse primer concentration; varying the probe, forward, and reverse primers concentrations in any way; varying the thermal cycling program; varying the PCR master mix; incorporating into the assay some fraction of probes that lack fluorophores; or incorporating into the assay any hybridization-based competitive inhibitors to probe binding, such as blocking oligomer nucleotides, peptide nucleic acids, and locked nucleic acids. The invention incorporates the use of these methods adjusting probe fluorescence intensity, or any other methods for adjusting probe fluorescence intensity, used either by themselves or in any combination.

Higher-plex Reactions

One method of the invention involves performing higher-plex assays with a single probe color (i.e. fluorophore). As described above, probe fluorescent intensities can be adjusted by a variety of means such that each intensity level uniquely identifies a DNA target. For example, targets T1, T2, T3, and T4 might be uniquely identified by intensity levels I1, I2, I3, and I4. Not intending to be bound by theory, the maximum number of intensity levels possible for unique identification of targets is related to the resolution of the different intensity levels--that is the spread of intensities for each particular probe compared to the separation between the average intensities of the probes--and it is also related to the intensity of the empty droplets that tends to grow with increasing numbers of probes. The number of intensity levels can be 0, or 1, or 2, or 3, or 4, or up to 10, or up to 20, or up to 50, or up to 100. The number of intensity levels can be higher than 100. In the examples show below, as many as three intensity levels are demonstrated.

Another method of the invention involves performing higher-plex assays using multiple different probe colors (i.e. fluorophores). As above for the monochromatic multiplexing assay, for each color probe, multiple targets can be identified based on intensity. Additionally, multiple colors that are spectrally separable can be used simultaneously. For example, a single droplet might contain four different probes for measuring four different targets. Two probes might be of color A with different intensities (say, A1 and A2), and the other two probes of color B with different intensities (say B1 and B2). The corresponding targets are T1, T2, T3, and T4 for A1, A2, B1, and B2 respectively. If a droplet shows an increase in fluoresce in color A, the droplet therefore contained either targets T1 or T2. Then, based on the fluorescence intensity of color A, the target could be identified as T1 or the target could be identified as T2. If, however, a droplet shows an increase in fluorescence in color B, the droplet therefore contained either targets T3 or T4. Then, based on the fluorescence intensity of color B, the target could be identified as T3 or the target could be identified as T4. Not intending to be bound by theory, the maximum number of different colors possible is limited by spectral overlap between fluorescence emission of the different fluorophores. The maximum number of colors can be 1, or 2, or 3, or 4, or up to 10, or up to 20. The maximum number of colors can be higher than 20. In the demonstrations that follow, the largest number of colors is two.

Another method of the invention involves performing higher-plex assays using multiple different probe colors (i.e. fluorophores), however unlike the strategy above where each target is identified by single type of probe with a unique color and intensity, instead in this method a single target may be identified by multiple probes that constitute a unique signature of both colors and intensities. For example, a single droplet might contain four different probes for measuring three different targets (say, T1, T2, and T3). Two probes might be of color A (say, A1, and A2), and two probes might be of color B (say, B1 and B2). T1 is measured by probe A1, T2 is measured by probe B1, but T3 is measured by both probes A2 and B2. Thus, when a droplet contains T1 only increased fluorescence appears in color A. When a droplet contains T2 only increased fluorescence appears in

color B. However when a droplet contains T3, increased fluorescence appears in both colors A and B.

Generally, without wishing to be constrained by theory, the above three methods for higher-plex dPCR are simplest to implement under conditions of terminal dilution, that is when the probability of multiple different target molecules co-occupying the same droplet is very low compared to the probability of any single target occupying a droplet. With multiple occupancy arises the complexity of simultaneous assays competing within the same reaction droplet, and also complexity of assigning the resulting fluorescence intensity that involves a combination of fluorescence from two different reaction products that may or may not be equal to the sum of the two fluorescence intensities of the individual reaction products. However, methods of the invention can accommodate these complications arising from multiple occupancy.

Methods of the invention for higher-plex reactions also include methods for primer and probe pairing. In the simplest case targets are unlikely to reside on the same DNA fragments, such as when targets are from different cells; or when targets are from different chromosomes within a single cell type; or when targets are distant from each other within a single chromosome such that they become physically separated during DNA fragmentation; or when targets are very close to each other within a chromosome, but nevertheless become separated by targeted cleavage of the DNA, such as by restriction enzyme digestion; or for any other reason. In such cases each probe can be paired with a single set of primers (forward and reverse). However, in other cases the target regions might frequently reside on the same DNA fragments, for example when targets reside within the same codon, or for any other reason. In such cases, a single set of primers might serve for multiple probes (for an example, see Pekin et al.).

Higher multiplex reactions can be performed to distinguish the haplotypes of two SNPs. For example, assume that at position one there can be genotypes A or A' and at position two there can be genotypes B or B'. In a diploid genome four unique haplotypes are possible (A,B; A,B'; A',B; and A',B'). If for example A' and B' represent drug resistant mutations for infection, it is often the case that A'B and AB' are less severe and treated differently than A'B' which represents a significant drug resistance that must be treated with extreme care. Digital PCR with intensity discrimination is ideally suited for identifying low prevalence of A'B' in a background of mixtures of the other three haplotypes. Haplotyping information is also important for construction of haplotypes in HLA. One way that the present example can be constructed is by assay design such that color one is used for A and is of high or low intensity indicative of allele A or A' respectively and color two is used for B and is of high or low intensity respectively indicative of B or B'. Populations of [color1,color2] corresponding to [Low, Low] would be a measure of an allele of AB and [high, low] allele A'B and an allele of [A'B'] will be readily distinguishable as [high, high] even in a background that is predominately a mixture of A'B and AB'. See FIG. 22. In some cases it will be advantageous to start by encapsulating into the droplets long single molecules of nucleic acid that contain both A and B SNP location and in other cases it will be desirable to start by encapsulating single cells, bacteria or other organism within the droplets prior to releasing the nucleic acid from the organism. In still other embodiments the multiplex intensity detection of multiple simultaneous targets can be used as surrogate markers for multiple types of binding interactions or labeling of target materials. This technique is also not limited to single molecule detection and can be used for haplotype detection in single cells (e.g., bacteria, somatic cells, etc.). In single cell analysis, a sorting step may be applied prior to haplotyping.

5-Plex Assay for Spinal Muscular Atrophy

An aspect of the invention was reduced to practice in an example demonstration of the quantitation of several

One embodiment of the invention is a 5-plex assay for SMA diagnostics. The 5-plex assay quantifies common genetic variants impacting SMA including two copy number assays for the SMN1 and SMN2 genes with BCKDHA as a reference, and a SNP assay for the c.815A>G mutation. Two differently colored fluorophores, FAM and VIC, were used to uniquely identify each of the assays. The probes for SMN1 and SMN2 contained only FAM, and for c.815A only VIC. However, mixtures of VIC and FAM-labeled probes were used for BCKDHA and c.815G. The use of VIC and FAM fluorophores in this example does not limit the invention, rather the 5-plex assay can be used with any spectrally separable fluorophores compatible with the TaqMan assay, or any other fluorogenic hybridization-based probe chemistries. For validating the assay, a model chromosome was synthesized containing a single target region for each of the different primer/probe pairs. EcoRV restriction sites flanked each target, allowing separation of the fragments.

In another method of the invention, the different populations were sufficiently well resolved to allow droplets within each population to be counted by integration across rectangular boundaries. The boundaries were positioned at mid-sections between neighboring peaks. The methods of the invention are not constrained to rectangular boundaries, or to specific boundary locations between peaks. Rather, any closed or unclosed boundary condition can suffice. Boundary conditions do not need to be "binary" either, in the sense that weighted integrations can also be performed across the boundaries to arrive at droplet counts. The peak position of each cluster varied by no more than 2% from run to run after normalization to the intensity of the empty droplets to

account for variations in detection efficiency (data not shown). Hence, once identified, the same boundaries for integration could be reused between samples. The methods of the invention are not limited to fixed boundary positions. Dynamic population identification and boundary selection in between samples or studies is anticipated. Twenty different patient samples from the Coriell cell repositories were analyzed with this assay: 4 afflicted with SMA, 1 SMA carrier, and 15 negative controls. Assay results are shown in FIG. 14b. Gene copy number was calculated as before, as the ratio of occupancies derived from the number of target droplets vs. reference droplets. Like the copy number measurement in FIG. 11, each assay yielded ratios very close to the expected integer values, but when all of the patient data was plotted as actual ratio vs. expected integer ratio a small systematic deviation from the ideal slope of 1 was observed. Measured slopes were 0.92, 0.92, and 0.99 for SMN1, SMN2, and c.815A respectively. For clarity, the data in FIG. 14b was scaled to the ideal slope of 1.

The measured genotypes of the different patients were consistent with their disease conditions (unafflicted, carrier, or afflicted). The patients afflicted with SMA each had zero copies of SMN1 (numbers SMA 1-4 in FIG. 14b), the carrier had just one copy, and the negative controls all had two or three copies (numbers 1-15). Three unrelated individuals (numbers 6, 8, and 9) had three copies of SMN1, occurring at a rate of 20% which is similar to a previous report for healthy individuals. Variability in SMN1 copy number is not surprising since it lies within an unstable region of chromosome 5q13. A larger variety of SMN2 copy numbers was observed. One to two copies were most common in the control group, although one individual had zero copies, a distribution consistent with expectations for normal individuals. The SMA carrier and afflicted patients had elevated copy numbers of SMN2 on average: 5 for the carrier, two afflicted with 3 copies, and the others with 2 copies. The afflicted patients were all diagnosed as SMA Type I, the most severe form, based on clinical observations according to the Coriell repository. The strong genotype/phenotype correlation between SMN2 copy number and disease severity suggests that the two individuals with three copies of SMN2 might have an improved Type II prognosis, especially for the patient SMA 1 who had survived to three years at the time of sampling, much beyond the typical maximum life expectancy for SMA Type I of 2 years. However there remains reluctance to predict disease outcome based on SMN2 copies alone since other less well characterized or unknown modifying genes may impact prognosis and because not all SMN2 copies may be complete genes. Furthermore some Type I patients have begun surviving longer in newer clinical settings. Hence, with little clinical information regarding the patients available to us, we can conclude that our SMN2 assay results were consistent with broad expectations for disease severity.

The SNP assay revealed that all patients carried the normal c.815A genotype and no instances of c.815G were observed. The mutation is relatively rare and hence was not expected to appear in a small patient panel. Of interest, however, was the presence of an apparent extra gene fragment in two unrelated individuals that was uncovered with the SNP assay. The c.815A>G assay does not discriminate between SMN1 and SMN2 due to their high sequence similarity, and hence the total copies of c.815A and G should equal the sum of the copies of SMN1 and SMN2. This was true for all patients except for healthy patients number 1 and 2, both of whom had one extra copy of c.815A. c.815 lies on exon 6, and the SNP that discriminates between the SMN1 and SMN2 genes lies on exon 7, hence the extra genes may be fragments of SMN1 lacking exon 7. This seems reasonable because the deletion of exon 7 is the common mutation causing 95% of cases of SMA (reviewed by Wirth et al.) and it is carried by 1/40 to 1/60 adults. Thus these patients might have been typical carriers of SMA but for the acquisition of at least one compensating healthy copy of SMN1 on the same chromosome.

9-Plex Assay for Spinal Muscular Atrophy

A 9-plex assay for certain SMA related targets was also demonstrated with just two colors (probes containing FAM and VIC fluorophores). Aside from the optimized primer and probe concentrations, assay conditions and experimental procedures were identical to the 5-plex assay above. FIG. 15a shows the various droplet populations in 2-D histograms before optimization of probe concentrations. The identity of the different targets is shown on the figure itself. As one method of the invention, the identification of the different populations was made as before, by selective exclusion and/or addition of one or more assays. Most of the populations were already well resolved, with the exception of the probe for the c.815A genotype that was in close proximity with the cluster corresponding to empty droplets. After three iterations of optimization of probe concentrations, all of the target populations were well resolved from each other, and well resolved from the empty droplets (FIG. 15b). Three methods of the invention were highlighted in this demonstration: (1) nine DNA targets were uniquely identified in a two-dimensional histogram, far beyond the capabilities of conventional qPCR; (2) target DNA molecules were distinguished on the basis of some combination of both color and intensity arising from one or multiple probes against the same target; and (3) the relative positions of the target molecules within the histogram were adjusted by varying the probe concentrations to optimize the pattern of colors and intensities for increased resolution amongst the various droplet populations.

As one method of the invention, different droplet populations were identified by selective addition or exclusion of assays in the examples above. However the invention is not limited to this method alone. Rather, any method for population assignments known to those in the art are considered. Methods of the invention include any method that can cause an identifiable displacement, appearance, or disappearance of one or more populations within the histograms including changing the probe and primer concentrations together, either by the same factor or by different factors; changing the probe concentration alone; changing the primer concentrations alone; changing the thermal cycling conditions; and changing the master mix composition. Another method of the invention takes advantage of prior knowledge of the position of an assay within a histogram to assist assignment.

Multiplexing Capacity

The level of multiplexing demonstrated in the preceding SMA example was 9.times., significantly exceeding the maximum practicable number with qPCR. Without wishing to be constrained by theory, the two main limitations are the resolution between assays and the increasing fluorescence intensity of empty droplets with higher loading of probes. A method of the invention involves optimizing the pattern of colors and intensities of the different probes for maximum multiplexing while still achieving adequate specificity for each individual reaction. Although rectangular arrays of droplet populations were demonstrated for the 5- and 9-plex reactions, another desirable pattern is the tight-packed hexagonal array. However the invention is not constrained to any particular array strategy.

Adding extra colors would increase the capability even further, however with some diminishing returns because the fluorescence of the empty droplets would continue to rise. The capacity could be yet further increased with better probes yielding larger differential signals, such as hybrid 5'-nuclease/molecular beacon probes that reduce background by contact quenching yet exhibit the bright signals typical of free unquenched fluorophores. With such improvements multiplexing capacity exceeding 50.times. can be envisioned.

Combined Multiplexing with Optical Labeling

Using droplet-based microfluidics, multiple targets can also be measured simultaneously by a different method.

According to the alternative method, primers and probes can be loaded individually into droplets along with an optical label to uniquely identify the assay. Typically the optical label is a fluorophore, or a combination of different fluorophores, that are spectrally distinct from the probe fluorophore. Various different types of droplets, each containing different assays that are uniquely identified by different optical labels, can be mixed into a "library" of droplets. Then, according to methods of the invention above, library droplets are merged one-to-one with droplets containing template DNA. After thermal cycling, some droplets that contain template DNA will exhibit brighter fluorescence at the emission wavelengths of the probes. The specific target DNA molecules giving rise to these PCR(+) signals are subsequently identified by the optical probes. In one study, the six common mutations in KRAS codon 12 were screened in parallel in a single experiment by one-to-one fusion of droplets containing genomic DNA with any one of seven different types of droplets (a seven-member library), each containing a TaqMan.RTM. probe specific for a different KRAS mutation, or wild-type KRAS, and an optical code.

In one method of the invention, optical labeling can be combined with the various methods for multiplexing dPCR already incorporated into this invention. For example, a single optical label might code for the entire 5-plex SMA assay, above, instead of just a single assay as in the KRAS example above. In this manner, other optical labels might code for different screening assays for newborn infants. According to other methods of the invention, above, a single DNA sample from an infant could then be analyzed with all of the assays simultaneously by merging droplets containing the DNA one-to-one with library droplets containing the optically encoded assays.

As an example of combining multiplexing with optical labels, a so called 3.times.3.times.3 combination multiplex reaction with optical labeling was demonstrated (3.times.3 optical labeling with two fluorophores, each encoding a triplex assay, for a total of 27-plex). Two fluorophores were employed for optical labeling, Alexa633 and CF680 (excited by a 640 nm laser), with three intensity levels each producing nine total optical labels. As before with the 5- and 9-plex assays for SMA, TaqMan assays were used with FAM and VIC fluorophores (excited by a 488 nm laser). The fluorescence from the FAM and VIC fluorophores were recorded simultaneously with the fluorescence from the optical labels, requiring modifications to the optical layout of the instrumentation described for the SMA assay (the optical schematic for two-laser excitation and 4-color detection is shown in entirety in FIG. 16). Also, co-flow microfluidics were used in this example (the use of co-flow based microfluidics for this application is one of the methods of the invention described above). In this case, the template DNA was introduced into the chip in one flow, and the PCR master mix, the primers and probes for one triplex assay, and the unique composition of fluorophores for the optical label were introduced into the chip in another flow simultaneously. The two flow streams converged in a fluidic intersection upstream from the droplet forming module, and thus each droplet formed contained the contents of both flow streams. Methods to implement co-flow microfluidics are well known to those in the art. The droplets were collected, and then the procedure was repeated with the next triplex assay and optical label. The procedure was repeated a total of nine times, once for each pair of assays and optical labels. All of the droplets were collected into a single PCR tube and thermally cycled off chip. The mixture of thermally cycled droplets was reinjected into the same read-out chip as used for the SMA assay, above, and the fluorescence intensities of the assays from all four fluorophores was recorded.

FIG. 17 shows the cumulative results from all droplets in the 3.times.3.times.3 assay using co-flow microfluidics. The figure shows two 2-D histograms of droplet fluorescence intensities, the histogram on the left from all of the optical labels, and the histogram on the right from the assays. Standard methods were used to compensate for spectral overlap. The histograms are shown as a heat maps, with hotter colors designating larger numbers of

droplets. Nine different clusters of droplets were clearly evident in the histogram of the optical labels, corresponding to each of the nine different optical labels: there is a small group of four clusters at the bottom left corner of the histogram, corresponding to optical labels with the lowest fluorescent intensities; and there are five clusters appearing as linear streaks at the higher intensities. The droplet clusters were less distinct in the histogram for the assay, but this was as expected because the droplets shown contained all of the triplex assays. The individual assays became clearly distinct once a single type of assay was selected by using the optical labels, as follows.

Methods of the invention involve selecting individual populations of droplets all containing the same optical labels, or groups of optical labels. In some methods of the invention, boundaries of fluorescence intensity were used to specify populations. In the example shown here, a rectangular boundary was used specifying the minimum and maximum fluorescence intensities for each fluorophore. However the methods of the invention are not restricted to rectangular boundaries. Any boundary, closed or unclosed, can be employed. Furthermore, according to methods of the invention, selections of droplet populations can be made by any method, and is not restricted to threshold-based methods such as boundary selection.

FIG. 18A shows the droplet fluorescence intensities for the assay (right histogram) when only one optical label was selected (left histogram). The lines overlaid on the histogram of the optical labels identify the rectangular boundary used to select just the optical label with the lowest fluorescence for both fluorophores. Both histograms showed only the droplets that were selected. After selection, four distinct clusters of droplets appeared in the assay histogram, three for the different assays (in this case, assays for SMN1, SMN2, and TERT, where TERT is another common reference gene) and one for the empty droplets. The copy numbers for SMN1 and SMN2 were measured by the same methods of the invention as described above for the 5-plex SMA assay, with values of 1.8 and 0.94 close to the expected values of 2 and 1, respectively. The same assay was encoded with two other optical labels, and their selections are shown in FIGS. 18B and C. Similar results were achieved, with an overall measurement of 1.9 ± 0.1 and 0.9 ± 0.1 copies of SMN1 and SMN2 respectively, showing the measurement to be accurate within experimental uncertainty.

FIGS. 19A, B, and C show optical label selections for a different assay (TERT, c.5C in the SMN1 gene, and BCKDHA (labeled E1a in the figure)). In each case four distinct clusters also appeared, and by the same methods of the invention above, accurate measurements of gene copy number were made for c.5C and BCKDHA, referenced to TERT, of 2.9 ± 0.1 and 2.0 ± 0.2 compared to 3 and 2, respectively. FIGS. 20A, B, and C show optical label selections for a third assay (TERT, c.88G in the SMN1 gene, and RNaseP, where RNaseP is a common reference gene). Accurate gene copy numbers of 2.1 ± 0.1 were measured for both c.88G and RNaseP, referenced to TERT, compared to the expected value of 2.

In summary, the demonstration here shows use of nine different optical labels to enable independent measurement of three triplex assays in a single experiment. Although some of the optical labels encoded for redundant assays in this example (there were only three different assays despite having nine optical labels), the invention is not constrained to any particular formatting of assays and optical labels. Embodiments of the invention include formats where all of the assays are the same across all of the optical labels; where none of the assays are the same across all of the optical labels; where some of the assays are the same across all of the optical labels; where some of the assays have greater plexity than others across all of the optical labels; where all of the assays have the same plexity across all of the optical labels; and any other arrangements of assays across all of the optical labels are considered.

Although solely triplex assays were used in the example demonstration here, the invention is not constrained to use of triplex assays with optical labels. Embodiments of the invention include plexities of the following amounts when used with optical labels: single plex, duplex, triplex, 4-plex, up to 10-plex, up to 20-plex, up to 50-plex, and up to 100-plex. Embodiments of the invention also include plexities exceeding 100 when used with optical labels.

Another method of the invention involves the use of droplet merging, instead of co-flow, for combining multiplexing with optical labels. A demonstration using droplet merging was performed with the same 3.times.3.times.3 assay as in the preceding example with co-flow. The assays (probes and primers) combined with their unique optical labels were first encapsulated into droplets along with the PCR master mix. Subsequently, according to methods of the invention described above, a library containing a mixture of droplets from all nine optically labeled assays was merged one-to-one with droplets containing template DNA from the same patient as in the preceding example. As another method of the invention, the droplet merge was performed using a lambda-injector style merge module, as described in U.S. Provisional Application Ser. No. 61/441,985, incorporated by reference herein. Aside from the differences between co-flow and merge, the assays and experimental procedures were identical to those above for the co-flow experiment. FIG. 21 shows 2-D histograms of droplet fluorescence intensity for the optical labels and the assays that are similar to those in FIGS. 17-20. As in the case for co-flow, upon selection of droplets containing individual optical labels, the expected distinct clusters of droplets corresponding to each assay were clearly evident. Furthermore for each assay the measured gene copy number matched or very nearly matched the expected values within experimental uncertainty (See Table 1).

TABLE-US-00002 TABLE 1 Gene copy number measurements from the 3 .times. 3 .times. 3 assay. Measured Expected Gene or genotype copy number copy number SMN1 1.98 .+-. 0.09 2 SMN2 0.99 .+-. 0.04 1 c.5C in SMN1 3.01 .+-. 0.06 3 c.88G in SMN1 2.15 .+-. 0.08 2 BCKDHA 2.00 .+-. 0.05 2 RNaseP 2.11 .+-. 0.16 2

Although methods of the invention include using either microfluidics with co-flow or droplet merging, the invention is not limited in this regard. Any fluidic method capable of generating optically labeled droplets that also contain fluorogenic DNA hybridization probes are considered. For example, other embodiments well known in the art are mixing optical labels and assays in the macrofluidic environment before injection into a droplet generating chip; and mixing optical labels and assays thoroughly upstream from the droplet forming module in dedicated mixing modules, such as with a serpentine mixer.

Data Analysis

One method of the invention involves histogram-based data presentation and analysis for identifying and characterizing populations of statistically similar droplets that arise from unique probe signatures (color and intensity), and for discriminating one population of droplets from the others. Another method of the invention

involves histogram-based data presentation and analysis for identifying and selecting populations of droplets based on unique signatures from optical labels. Examples of one and two-dimensional histograms have been provided for these methods, but the invention is not limited in this regard. As described above, it is anticipated that greater numbers of colors will be used for both multiplexing and for optical labels. Hence, embodiments of the invention include histograms of dimensionality greater than two, such as 3, or 4, or up to 10, or up to 20. Histograms of dimensionality greater than 20 are also incorporated into the invention.

Another method of the invention involves the selection of droplets within histograms, either for counting, or for assay selection as in the use of optical labels, or for any other purpose. Methods of the invention include selections by boundaries, either closed or unclosed, of any possible shape and dimension. Methods of the invention also include selections of droplets that exhibit fluorescence from single types of fluorophores, or from multiple types of fluorophores, such as arising from multiple probes against a common DNA target.

Polymerase Error Correction

For applications requiring very high sensitivity, such as searching for rare mutations amidst an abundance of wild-type DNA, false positive results can arise from errors from the DNA polymerase itself. For example, during one of the early thermal cycles the polymerase might synthesize the mutant strand of DNA from a wild-type template. This type of error is most likely to occur when the difference between the mutant and the wild-type is very small, such as single nucleotide polymorphism (SNP). In this method of the invention, each droplet contains only a single target nucleic acid, if any at all. In the preferred embodiment, this is accomplished under the conditions of terminal dilution. Droplets that contain amplification products that are a wild-type of the target are detected based on emission from the fluorophore that is released from the probe that hybridizes to the wild-type of the target. Droplets that contain the variant of the target are detected based on emission from the fluorophore that is released from the probe that hybridizes to the variant of the target. Since each droplet starts with only a single nucleic acid molecule, the resultant amplification products in each droplet are either homogeneous for the target or homogenous for the variant of the target.

However, certain droplets will contain a heterogeneous mixture of both target and target variant due to polymerase errors during the PCR reaction. Error rates in PCR vary according to the precise nucleic acid sequence, the thermostable enzyme used, and the in vitro conditions of DNA synthesis. For example, the error frequency (mutations per nucleotide per cycle) during PCR catalyzed by the thermostable *Thermus aquaticus* (Taq) DNA polymerase vary more than 10-fold, from 2×10^{-4} to $<1 \times 10^{-5}$. Eckert et al. (Genome Res. 1:17-24, 1991), the content of which is incorporated by reference herein in its entirety. Polymerase-mediated errors at a frequency of 1 mutation per 10,000 nucleotides per cycle are an important consideration for any PCR application that begins with a small amount of starting material (e.g., less than a total of 10,000 nucleotides of target DNA) or that focuses on individual DNA molecules in the final PCR population.

The proportion of DNA molecules that contain sequence changes is a function of the error rate per nucleotide per cycle, the number of amplification cycles and the starting population size. The population of altered DNA molecules arises during PCR from two sources: (1) new errors at each PCR cycle; and (2) amplification of DNA molecules containing errors from previous cycles. The formula $f=np/2$ describes the average mutation frequency (f) for PCR amplification as a function of the polymerase error rate per nucleotide per cycle (p) and the number of cycles (n), assuming that p is constant at each cycle. Due to the exponential nature of PCR, the occurrence of an early error can increase the final error frequency above the average described by $f=np/2$, because the variant

DNA molecule will be amplified with each cycle, resulting in populations with a larger than average number of variants.

A polymerase error that converts a wild-type of the target to a variant of the target during an early round of amplification results in a heterogeneous population of target and target variant in a droplet, and may lead to a droplet being incorrectly identified as containing a variant of the target, i.e., a false positive. Such false positives greatly impact the validity and precision of digital PCR results.

Methods of the invention are able to detect which droplets contain a heterogeneous population of molecules and are able to exclude those droplets from analysis. As droplets containing amplified product flow in a channel through the detector module, the module is able to detect the fluorescent emission in each droplet. Droplets that produce only a single signal are classified as droplets that contain a homogeneous population of target. Since probes that hybridize to the wild-type of the target have a different fluorophore attached than probes that hybridize to a variant of the wild-type of the target, methods of the invention can classify each droplet as containing either a homogeneous population of amplicons of the target or a homogeneous population of amplicons of the variant of the target.

Droplets that produce two signals are classified as droplets that contain a heterogeneous population of molecules. Since each droplet started with at most a single target nucleic acid, a droplet that includes amplification products that are both amplicons of the target and amplicons of a variant of the target are droplets in which the variant of the target was produced by a polymerase error during the PCR reaction, most likely a polymerase error during an early cycle of the PCR reaction. Such droplets are detected and excluded from analysis.

Analysis

Analyze is then performed on only the droplets that contain a homogeneous population of molecules. The analysis may be based on counting, i.e., determining a number of droplets that contain only wild-type target, and determining a number of droplets that contain only a variant of the target. Such methods are well known in the art. See, e.g., Lapidus et al. (U.S. Pat. Nos. 5,670,325 and 5,928,870) and Shuber et al. (U.S. Pat. Nos. 6,203,993 and 6,214,558), the content of each of which is incorporated by reference herein in its entirety.

Generally, the presence of droplets containing only variant is indicative of a disease, such as cancer. In certain embodiments, the variant is an allelic variant, such as an insertion, deletion, substitution, translocation, or single nucleotide polymorphism (SNP).

Biomarkers that are associated with cancer are known in the art. Biomarkers associated with development of breast cancer are shown in Erlander et al. (U.S. Pat. No. 7,504,214), Dai et al. (U.S. Pat. Nos. 7,514,209 and 7,171,311), Baker et al. (U.S. Pat. No. 7,056,674 and U.S. Pat. No. 7,081,340), Erlander et al. (US 2009/0092973). The contents of the patent application and each of these patents are incorporated by reference herein in their entirety. Biomarkers associated with development of cervical cancer are shown in Patel (U.S. Pat. No. 7,300,765), Pardee et al. (U.S. Pat. No. 7,153,700), Kim (U.S. Pat. No. 6,905,844), Roberts et al. (U.S. Pat. No. 6,316,208), Schlegel (US 2008/0113340), Kwok et al. (US 2008/0044828), Fisher et al. (US 2005/0260566), Sastry et al. (US 2005/0048467), Lai (US 2008/0311570) and Van Der Zee et al. (US 2009/0023137). Biomarkers associated with development of vaginal cancer are shown in Giordano (U.S. Pat.

No. 5,840,506), Kruk (US 2008/0009005), Hellman et al. (Br J Cancer. 100(8):1303-1314, 2009). Biomarkers associated with development of brain cancers (e.g., glioma, cerebellum, medulloblastoma, astrocytoma, ependymoma, glioblastoma) are shown in D'Andrea (US 2009/0081237), Murphy et al. (US 2006/0269558), Gibson et al. (US 2006/0281089), and Zetter et al. (US 2006/0160762). Biomarkers associated with development of renal cancer are shown in Patel (U.S. Pat. No. 7,300,765), Soyupak et al. (U.S. Pat. No. 7,482,129), Sahin et al. (U.S. Pat. No. 7,527,933), Price et al. (U.S. Pat. No. 7,229,770), Raitano (U.S. Pat. No. 7,507,541), and Becker et al. (US 2007/0292869). Biomarkers associated with development of hepatic cancers (e.g., hepatocellular carcinoma) are shown in Horne et al. (U.S. Pat. No. 6,974,667), Yuan et al. (U.S. Pat. No. 6,897,018), Hanausek-Walaszek et al. (U.S. Pat. No. 5,310,653), and Liew et al. (US 2005/0152908). Biomarkers associated with development of gastric; gastrointestinal, and/or esophageal cancers are shown in Chang et al. (U.S. Pat. No. 7,507,532), Bae et al. (U.S. Pat. No. 7,368,255), Muramatsu et al. (U.S. Pat. No. 7,090,983), Sahin et al. (U.S. Pat. No. 7,527,933), Chow et al. (US 2008/0138806), Waldman et al. (US 2005/0100895), Goldenring (US 2008/0057514), An et al. (US 2007/0259368), Guilford et al. (US 2007/0184439), Wirtz et al. (US 2004/0018525), Filella et al. (Acta Oncol. 33(7):747-751, 1994), Waldman et al. (U.S. Pat. No. 6,767,704), and Lipkin et al. (Cancer Research, 48:235-245, 1988). Biomarkers associated with development of ovarian cancer are shown in Podust et al. (U.S. Pat. No. 7,510,842), Wang (U.S. Pat. No. 7,348,142), O'Brien et al. (U.S. Pat. Nos. 7,291,462, 6,942,978, 6,316,213, 6,294,344, and 6,268,165), Ganetta (U.S. Pat. No. 7,078,180), Malinowski et al. (US 2009/0087849), Beyer et al. (US 2009/0081685), Fischer et al. (US 2009/0075307), Mansfield et al. (US 2009/0004687), Livingston et al. (US 2008/0286199), Farias-Eisner et al. (US 2008/0038754), Ahmed et al. (US 2007/0053896), Giordano (U.S. Pat. No. 5,840,506), and Tchagang et al. (Mol Cancer Ther, 7:27-37, 2008). Biomarkers associated with development of head-and-neck and thyroid cancers are shown in Sidransky et al. (U.S. Pat. No. 7,378,233), Skolnick et al. (U.S. Pat. No. 5,989,815), Budiman et al. (US 2009/0075265), Hasina et al. (Cancer Research, 63:555-559, 2003), Kebebew et al. (US 2008/0280302), and Ralhan (Mol Cell Proteomics, 7(6):1162-1173, 2008). The contents of each of the articles, patents, and patent applications are incorporated by reference herein in their entirety. Biomarkers associated with development of colorectal cancers are shown in Raitano et al. (U.S. Pat. No. 7,507,541), Reinhard et al. (U.S. Pat. No. 7,501,244), Waldman et al. (U.S. Pat. No. 7,479,376); Schleyer et al. (U.S. Pat. No. 7,198,899); Reed (U.S. Pat. No. 7,163,801), Robbins et al. (U.S. Pat. No. 7,022,472), Mack et al. (U.S. Pat. No. 6,682,890), Tabiti et al. (U.S. Pat. No. 5,888,746), Budiman et al. (US 2009/0098542), Karl (US 2009/0075311), Arjol et al. (US 2008/0286801), Lee et al. (US 2008/0206756), Mori et al. (US 2008/0081333), Wang et al. (US 2008/0058432), Belacel et al. (US 2008/0050723), Stedronsky et al. (US 2008/0020940), An et al. (US 2006/0234254), Eveleigh et al. (US 2004/0146921), and Yeatman et al. (US 2006/0195269). Biomarkers associated with development of prostate cancer are shown in Sidransky (U.S. Pat. No. 7,524,633), Platica (U.S. Pat. No. 7,510,707), Salceda et al. (U.S. Pat. No. 7,432,064 and U.S. Pat. No. 7,364,862), Siegler et al. (U.S. Pat. No. 7,361,474), Wang (US 7,348,142), Ali et al. (U.S. Pat. No. 7,326,529), Price et al. (U.S. Pat. No. 7,229,770), O'Brien et al. (U.S. Pat. No. 7,291,462), Golub et al. (U.S. Pat. No. 6,949,342), Ogden et al. (U.S. Pat. No. 6,841,350), An et al. (U.S. Pat. No. 6,171,796), Bergan et al. (US 2009/0124569), Bhowmick (US 2009/0017463), Srivastava et al. (US 2008/0269157), Chinnaiyan et al. (US 2008/0222741), Thaxton et al. (US 2008/0181850), Dahary et al. (US 2008/0014590), Diamandis et al. (US 2006/0269971), Rubin et al. (US 2006/0234259), Einstein et al. (US 2006/0115821), Paris et al. (US 2006/0110759), Condon-Cardo (US 2004/0053247), and Ritchie et al. (US 2009/0127454). Biomarkers associated with development of pancreatic cancer are shown in Sahin et al. (U.S. Pat. No. 7,527,933), Raitano et al. (U.S. Pat. No. 7,507,541), Schleyer et al. (U.S. Pat. No. 7,476,506), Domon et al. (U.S. Pat. No. 7,473,531), McCaffey et al. (U.S. Pat. No. 7,358,231), Price et al. (U.S. Pat. No. 7,229,770), Chan et al. (US

2005/0095611), Mitchl et al. (US 2006/0258841), and Faca et al. (PLoS Med 5(6):e123, 2008). Biomarkers associated with development of lung cancer are shown in Sahin et al. (U.S. Pat. No. 7,527,933), Hutteman (U.S. Pat. No. 7,473,530), Bae et al. (U.S. Pat. No. 7,368,255), Wang (U.S. Pat. No. 7,348,142), Nacht et al. (U.S. Pat. No. 7,332,590), Gure et al. (U.S. Pat. No. 7,314,721), Patel (U.S. Pat. No. 7,300,765), Price et al. (U.S. Pat. No. 7,229,770), O'Brien et al. (U.S. Pat. No. 7,291,462 and U.S. Pat. No. 6,316,213), Muramatsu et al. (U.S. Pat. No. 7,090,983), Carson et al. (U.S. Pat. No. 6,576,420), Giordano (U.S. Pat. No. 5,840,506), Guo (US 2009/0062144), Tsao et al. (US 2008/0176236), Nakamura et al. (US 2008/0050378), Raponi et al. (US 2006/0252057), Yip et al. (US 2006/0223127), Pollock et al. (US 2006/0046257), Moon et al. (US 2003/0224509), and Budiman et al. (US 2009/0098543). Biomarkers associated with development of skin cancer (e.g., basal cell carcinoma, squamous cell carcinoma, and melanoma) are shown in Roberts et al. (U.S. Pat. No. 6,316,208), Polsky (U.S. Pat. No. 7,442,507), Price et al. (U.S. Pat. No. 7,229,770), Genetta (U.S. Pat. No. 7,078,180), Carson et al. (U.S. Pat. No. 6,576,420), Moses et al. (US 2008/0286811), Moses et al. (US 2008/0268473), Dooley et al. (US 2003/0232356), Chang et al. (US 2008/0274908), Alain et al. (US 2008/0118462), Wang (US 2007/0154889), and Zetter et al. (US 2008/0064047). Biomarkers associated with development of multiple myeloma are shown in Coignet (U.S. Pat. No. 7,449,303), Shaughnessy et al. (U.S. Pat. No. 7,308,364), Seshi (U.S. Pat. No. 7,049,072), and Shaughnessy et al. (US 2008/0293578, US 2008/0234139, and US 2008/0234138). Biomarkers associated with development of leukemia are shown in Ando et al. (U.S. Pat. No. 7,479,371), Coignet (U.S. Pat. No. 7,479,370 and U.S. Pat. No. 7,449,303), Davi et al. (U.S. Pat. No. 7,416,851), Chiorazzi (U.S. Pat. No. 7,316,906), Seshi (U.S. Pat. No. 7,049,072), Van Baren et al. (U.S. Pat. No. 6,130,052), Taniguchi (U.S. Pat. No. 5,643,729), Insel et al. (US 2009/0131353), and Van Bockstaele et al. (Blood Rev. 23(1):25-47, 2009). Biomarkers associated with development of lymphoma are shown in Ando et al. (U.S. Pat. No. 7,479,371), Levy et al. (U.S. Pat. No. 7,332,280), and Arnold (U.S. Pat. No. 5,858,655). Biomarkers associated with development of bladder cancer are shown in Price et al. (U.S. Pat. No. 7,229,770), Orntoft (U.S. Pat. No. 6,936,417), Haak-Frendscho et al. (U.S. Pat. No. 6,008,003), Feinstein et al. (U.S. Pat. No. 6,998,232), Elting et al. (US 2008/0311604), and Wewer et al. (2009/0029372). The content of each of the above references is incorporated by reference herein in its entirety.

In certain embodiments, methods of the invention may be used to monitor a patient for recurrence of a cancer. Since the patient has already been treated for the cancer, the genetic profile and particular mutation(s) associated with that patient's cancer are already known. Probes may be designed that specifically hybridize to the region of the nucleic acid that contains the mutation(s) that is indicative of the cancer for which the patient was previously treated. A patient's sample (e.g., pus, sputum, semen, urine, blood, saliva, stool, or cerebrospinal fluid) may then be analyzed as described above to determine whether the mutant allele(s) is detected in the sample, the presence of which being indicative of recurrence of the cancer.

Droplet Sorting

Methods of the invention may further include sorting the droplets based upon whether the droplets contain a homogeneous population of molecules or a heterogeneous population of molecules. A sorting module may be a junction of a channel where the flow of droplets can change direction to enter one or more other channels, e.g., a branch channel, depending on a signal received in connection with a droplet interrogation in the detection module. Typically, a sorting module is monitored and/or under the control of the detection module, and therefore a sorting module may correspond to the detection module. The sorting region is in communication with and is influenced by one or more sorting apparatuses.

A sorting apparatus includes techniques or control systems, e.g., dielectric, electric, electro-osmotic, (micro-) valve, etc. A control system can employ a variety of sorting techniques to change or direct the flow of molecules, cells, small molecules or particles into a predetermined branch channel. A branch channel is a channel that is in communication with a sorting region and a main channel. The main channel can communicate with two or more branch channels at the sorting module or branch point, forming, for example, a T-shape or a Y-shape. Other shapes and channel geometries may be used as desired. Typically, a branch channel receives droplets of interest as detected by the detection module and sorted at the sorting module. A branch channel can have an outlet module and/or terminate with a well or reservoir to allow collection or disposal (collection module or waste module, respectively) of the molecules, cells, small molecules or particles. Alternatively, a branch channel may be in communication with other channels to permit additional sorting.

A characteristic of a fluidic droplet may be sensed and/or determined in some fashion, for example, as described herein (e.g., fluorescence of the fluidic droplet may be determined), and, in response, an electric field may be applied or removed from the fluidic droplet to direct the fluidic droplet to a particular region (e.g. a channel). In certain embodiments, a fluidic droplet is sorted or steered by inducing a dipole in the uncharged fluidic droplet (which may be initially charged or uncharged), and sorting or steering the droplet using an applied electric field. The electric field may be an AC field, a DC field, etc. For example, a channel containing fluidic droplets and carrier fluid, divides into first and second channels at a branch point. Generally, the fluidic droplet is uncharged. After the branch point, a first electrode is positioned near the first channel, and a second electrode is positioned near the second channel. A third electrode is positioned near the branch point of the first and second channels. A dipole is then induced in the fluidic droplet using a combination of the electrodes. The combination of electrodes used determines which channel will receive the flowing droplet. Thus, by applying the proper electric field, the droplets can be directed to either the first or second channel as desired. Further description of droplet sorting is shown for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to Raindance Technologies Inc.

Based upon the detected signal at the detection module, droplets containing a heterogeneous population of molecules are sorted away from droplets that contain a homogeneous population of molecules. Droplets may be further sorted to separate droplets that contain a homogeneous population of amplicons of the target from droplets that contain a homogeneous population of amplicons of the variant of the target.

Release of Target from Droplet

Methods of the invention may further involve releasing amplified target molecules from the droplets for further analysis. Methods of releasing amplified target molecules from the droplets are shown in for example in Link et al. (U.S. patent application numbers 2008/0014589, 2008/0003142, and 2010/0137163) and European publication number EP2047910 to RainDance Technologies Inc.

In certain embodiments, sample droplets are allowed to cream to the top of the carrier fluid. By way of non-limiting example, the carrier fluid can include a perfluorocarbon oil that can have one or more stabilizing surfactants. The droplet rises to the top or separates from the carrier fluid by virtue of the density of the carrier fluid being greater than that of the aqueous phase that makes up the droplet. For example, the perfluorocarbon oil used in one embodiment of the methods of the invention is 1.8, compared to the density of the aqueous phase of the droplet, which is 1.0.

The released amplified material can also be subjected to further amplification by the use of primers and secondary PCR primers. In this embodiment the primers in the droplet contain an additional sequence or tail added onto the 5' end of the sequence specific portion of the primer. The sequences for the tailed regions are the same for each primer pair and are incorporated onto the 5' portion of the amplicons during PCR cycling. Once the amplicons are removed from the droplets, another set of PCR primers that can hybridize to the tail regions of the amplicons can be used to amplify the products through additional rounds of PCR. The secondary primers can exactly match the tailed region in length and sequence or can themselves contain additional sequence at the 5' ends of the tail portion of the primer. During the secondary PCR cycling these additional regions also become incorporated into the amplicons. These additional sequences can include, but are not limited to adaptor regions utilized by sequencing platforms for library preparation and sequencing, sequences used as a barcoding function for the identification of samples multiplexed into the same reaction, molecules for the separation of amplicons from the rest of the reaction materials such as biotin, digoxin, peptides, or antibodies and molecules such as fluorescent markers that can be used to identify the fragments.

Briefly, a single-stranded nucleic acid (e.g., DNA or cDNA) is hybridized to oligonucleotides attached to a surface of a flow cell. The single-stranded nucleic acids may be captured by methods known in the art, such as those shown in Lapidus (U.S. Pat. No. 7,666,593). The oligonucleotides may be covalently attached to the surface or various attachments other than covalent linking as known to those of ordinary skill in the art may be employed. Moreover, the attachment may be indirect, e.g., via the polymerases of the invention directly or indirectly attached to the surface. The surface may be planar or otherwise, and/or may be porous or non-porous, or any other type of surface known to those of ordinary skill to be suitable for attachment. The nucleic acid is then sequenced by imaging the polymerase-mediated addition of fluorescently-labeled nucleotides incorporated into the growing strand surface oligonucleotide, at single molecule resolution.

What follows is experimental detail for the various experiments details above.

All TaqMan.RTM. primers and probes used here are listed in Table 2. Unless otherwise noted by reference in

the table, the primers and probes were designed with the "Custom TaqMan.RTM. Assay Design Tool" from Applied Biosystems Inc. (ABI) and procured through ABI (Carlsbad, Calif). Probes were labeled with 6-carboxyfluorescein (FAM, $\lambda_{\text{exc}} 494 \text{ nm}$, $\lambda_{\text{em}} 494 \text{ nm}$) or VIC.TM. (from ABI, $\lambda_{\text{exc}} 538 \text{ nm}$, $\lambda_{\text{em}} 554 \text{ nm}$).

TABLE-US-00003 TABLE 2 5-plex assay Target Assay Primers (5' to 3') Probe (5' to 3') conditions Ref
 SMN1 Copy number (f) AATGCTTTTAA- FAM-CAGGGTTTC*AGACAAA- 0.37.times. Anhuf
 CATCCATATAAAGCT MGBNFQ et al., 2003 (SEQ ID NO.: 1) (SEQ ID NO.: 3) (r) CCTTAATTAAAG-
 GAATGTGAGCACC (SEQ ID NO.: 2) SMN2 Copy number (f) AATGCTTTTAA- FAM-
 TGATTTTGTCTA*AAA- 0.76.times. Anhuf CATCCATATAAAGCT CCC-MGBNFQ et al., 2003 (SEQ
 ID NO.: 4) (SEQ ID NO.: 6) (r) CCTTAATTAAAG- GAATGTGAGCACC (SEQ ID NO.: 5) BCKDHA
 Copy number (f) CAACCTACTCTT- (FAM/VIC)-CAGGAGATGCCCG- FAM: 0.18.times. DiMatteo
 CTCAGACGTGTA CCCAGCTC-TAMRA VIC: 0.56.times. et al., 2008 (SEQ ID NO.: 7) (SEQ ID NO.: 9)
 (r) TCGAAGTGATCC- AGTGGGTAGTG (SEQ ID NO.: 8) c.815A > G SNP (f) TGCTGATGCTTT- (A)
 (FAM/VIC)-CATGAGTGG- 0.9.times. GGGAAGTATGTTA CTA*TCATAC-MGBNFQ (SEQ ID NO.:
 10) (SEQ ID NO.: 11) (r) TGTCAGGAAAAG- (G) FAM-ATGAGTGGCTG*TC- FAM: 0.9.times.
 ATGCTGAGTGATT ATAC-MGBNFQ (SEQ ID NO.: 12) (SEQ ID NO.: 13); VIC-CATGA-
 GTGGCTG*TCATAC- VIC: 0.45.times. MGBNFQ (SEQ ID NO.: 14) RNaseP Copy number Unknown
 unknown n/a Standard product, 4403326, ABI 5'-exonuclease genotyping assay design. Assay conditions in
 column 5 are specific to the multiplexed SMA assay. References: D. Anhuf, T. Eggermann, S. Rudnik-
 Schoneborn and K. Zerres, Hum Mutat., 2003, 22, 74-78; D. DiMatteo, S. Callahan and E. B. Kmiec, Exp
 Cell Res., 2008, 15, 878-886.

Target DNA

For some genetic targets, BCKDHA and SMN2, plasmid DNA was synthesized (GeneArt, Regensburg, Germany) containing the sequence spanning between the primers (see Table 2) and cloned into the GeneArt standard vector (2.5 kb). The target fragment was released from the cloning vector by restriction digestion with SfiI to avoid any DNA supercoiling that might affect the assay. For simplicity, these gene fragments are called "plasmid DNA" throughout the text. A string of different gene fragments was also synthesized (GeneArt) and cloned into the GeneArt standard vector for demonstration of multiplexed reactions, called an "artificial chromosome" in the text. In this case, the fragments were separated from each other by restriction digestion at flanking EcoRV sites. Human DNA was obtained in already purified form from cell lines (See Table 3; Coriell, Camden, N.J.) and fragmented before use with a K7025-05 nebulizer following manufacturer's instructions (Invitrogen, Carlsbad, Calif). DNA concentration was quantified by measuring absorbance at 260 nm on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Del.).

TABLE-US-00004 TABLE 3 Map of patient numbers used in the text to Coriell cell lines. Patient Coriell cell
 Patient number, Coriell cell number line cont'd. line, cont'd. 1 NA14638 11 NA13714 2 NA14637 12
 NA13712 3 NA14097 13 NA13709 4 NA14096 14 NA13707 5 NA14094 15 NA13705 6 NA14093
 SMA carrier NA03814 7 NA14092 SMA 1 NA03813 8 NA14091 SMA 2 NA00232 9 NA14090 SMA 3
 NA09677 10 NA13715 SMA 4 NA10684

Microfluidics

Microfluidic chips were manufactured by conventional soft lithography. Molding masters were fabricated by spin coating SU-8 negative photoresist (MicroChem Corp., Newton, Mass.) onto 6 inch silicon wafers and transferring the fluidic features from photomasks (CAD/Art Services, Bandon, Oreg.) by contact lithography with an OAI Hybralign Series 200 aligner (OAI, San Jose, Calif.). Chips contained channels with two depths: deep channels with low hydrodynamic resistance ($100. \pm .10 \mu\text{m}$) for transporting fluid from external ports to the functional regions of the chip, and shallow channels ($20. \pm .1 \mu\text{m}$) for droplet manipulation and detection. SU-8 photoresists 2100 and 2025 were used for deep and shallow channels respectively. Polydimethylsiloxane (PDMS) (Sylgard.RTM. 184, Dow Corning, Midland, Mich.) chips were molded from the negative masters within mold housings of custom design. Glass cover slides were permanently bonded to the fluidic side of the chips by surface activation in an AutoGlow.TM. oxygen plasma system (Glow Research, Phoenix, Ariz.) followed by immediate contact bonding. To create hydrophobic surfaces, the microfluidic channels were exposed for .about.2 min to 1H,1H,2H,2H-perfluorodecyltrichlorosilane (Alfa Aesar, Ward Hill, Mass.) dissolved in FC-3283 (3M Specialty Materials, St. Paul, Minn.) prepared as a mixture of 18 g silane in 100 μL solvent.

Two different microfluidic devices were used, one for droplet generation and the other for fluorescence readout after thermal cycling. The droplet generation chip created an emulsion of uniformly sized aqueous droplets of template DNA and PCR master mix that were suspended in an inert fluorinated oil with an emulsion stabilizing surfactant, called "carrier oil" from this point forward (REB carrier oil; RainDance Technologies, Lexington, Mass.). Droplets were generated in a cross-shaped microfluidic intersection, or "nozzle". As shown in FIG. 3a, under typical operation the aqueous phase flowed into the nozzle from the right (160 $\mu\text{L/hr}$), joining flows of the carrier oil from the top and bottom (750 $\mu\text{L/hr}$ of total oil), and producing 4 μL droplets at a rate of 11 kHz. The channel widths at the intersection measured 15 μm for the aqueous inlet, 12.5 for the oil inlets, and 15 μm widening to 40 μm at the outlet. Flow was driven by custom OEM pumps (IDEX Corporation, Northbrook, IL).

Approximately 25 μL of the PCR reaction mixture was collected as an emulsion from the droplet generation chip and thermally cycled in a DNA Engine (Bio-Rad, Hercules, Calif.). The reaction mixture contained 1. times. TaqMan.RTM. universal PCR master mix (Applied Biosystems, Carlsbad, Calif.), 0.2 mM dNTP (Takara Bio, Madison, Wis.), and various amounts of primer pairs and probes as described in the results. 1. times. assay concentration is defined as 0.2 μM probes with 0.9 μM primers. In all cases, when varied from the 1. times. concentration, the primers and probes were varied by the same amount. The cycler program included a 10 min hot start at 95.degree. C., and 45 cycles of 15 s at 95.degree. C. and 60 s at 60.degree. C.

The droplets became concentrated during off-chip handling because the carrier oil is more dense than the aqueous phase and drained down from the emulsion. Hence the droplets were reinjected into the readout chip as a tightly packed emulsion that required dilution prior to readout to properly distinguish one droplet from another. A "spacer" nozzle similar to the droplet generation nozzle above was used to inject uniform plugs of extra carrier oil between droplets immediately before readout. As shown in FIG. 3b, the droplet entrance into the nozzle tapered down into a constriction about the size of an individual droplet forcing the droplets to enter the nozzle in single file and consequently at a stable rate. Opposed flow of the carrier oil from the top and bottom channels separated the droplets uniformly. The channel leaving the spacer nozzle increased in width along the direction of flow, and the droplets were interrogated by laser induced fluorescence at the location along the channel where the width was smaller than or equal to the droplet diameter (marked with an arrow in FIG. 3b). The nozzle dimensions were 15 μm for the droplet entrance and exit, and 20 μm for the oil lines.

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